# THE
# BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

W. C. ALLEE, University of Florida
L. R. BLINKS, Stanford University
L. V. HEILBRUNN, University of Pennsylvania
A. K. PARPART, Princeton University
BERTA SCHARRER, University of Colorado
F. SCHRADER, Columbia University
H. B. STEINBACH, University of Minnesota
WM. RANDOLPH TAYLOR, University of Michigan
ALBERT TYLER, California Institute of Technology
JOHN H. WELSH, Harvard University
DOUGLAS WHITAKER, Stanford University
RALPH WICHTERMAN, Temple University

DONALD P. COSTELLO, University of North Carolina
*Managing Editor*

VOLUME 105
AUGUST TO DECEMBER, 1953

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year at the Lancaster Press, Inc., Prince and Lemon Streets, Lancaster, Pennsylvania.

Subscriptions and similar matter should be addressed to The Biological Bulletin, Marine Biological Laboratory, Woods Hole, Massachusetts. Agent for Great Britain: Wheldon and Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2. Single numbers $2.50. Subscription per volume (three issues), $6.00.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Massachusetts, between June 15 and September 1, and to Dr. Donald P. Costello, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina, during the remainder of the year.

Entered as second-class matter May 17, 1930, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

LANCASTER PRESS, INC., LANCASTER, PA.

# CONTENTS

PAGE

No. 1. August, 1953

Annual Report ..... 1
Anderson, John Maxwell
Structure and function in the pyloric caeca of Asterias forbesi ..... 47
Child, C. M.
Redox indicator patterns in relation to echinoderm exogastrulation. I. Oxidation patterns ..... 62
Foulkes, Robert Hugh
Regeneration of the anterior end of Aulophorus furcatus (Naididae) with special reference to effect of x-rays ..... 80
Goldstein, Lester
A study of the mechanism of activation and nuclear breakdown in the Chaetopterus egg ..... 87
Haxo, F. T., and K. A. Clendenning
Photosynthesis and phototaxis in Ulva lactuca gametes ..... 103
Hodgson, Edward S.
A study of chemoreception in aqueous and gas phases ..... 115
Love, Warner E.
The permeability of the erythrocyte-like cells of Phascolosoma gouldi .. 128
Nanney, David L.
Nucleo-cytoplasmic interaction during conjugation in Tetrahymena ... 133
Pettibone, Marian H.
Fresh-water polychaetous annelid, Manayunkia speciosa Leidy, from Lake Erie ..... 149
Rockstein, Morris
Some aspects of physiological aging in the adult worker honey bee .... 154
Rosenthal, Harold L.
Embryonic development of the lordotic and normal guppy, Lebistes reticulatus (Peters) ..... 160
Sacktor, Bertram, Gerard M. Thomas, John C. Moser and Donald I. Bloch
Dephosphorylation of adenosine triphosphate by tissues of the American cockroach, Periplaneta americana (L.) ..... 166
Schmidt, Edmond L., and Carroll M. Williams
Physiology of insect diapause. V. Assay of the growth and differentiation hormone of Lepidoptera by the method of tissue culture ..... 174
Schneiderman, Howard A., Melvin Ketchel and Carroll M. Williams
The physiology of insect diapause. VI. Effects of temperature, oxygen tension, and metabolic inhibitors on in vitro spermatogenesis in the Cecropia silkworm ..... 188

PAGE

ZACKS, SUMNER I., AND JOHN H. WELSH
Cholinesterase and lipase in the amoebocytes, intestinal epithelium and heart muscle of the quahog, Venus mercenaria ..... 200

No. 2. OCTOBER, 1953

ALLEN, ROBERT DAY
Fertilization and artificial activation in the egg of the surf-clam, Spisula solidissima ..... 213
VON BERTALANFFY, LUDWIG, AND W. J. PIROZYNSKI
Tissue respiration, growth, and basal metabolism ..... 240
DETHIER, V. G.
Summation and inhibition following contralateral stimulation of the tarsal chemoreceptors of the blowfly ..... 257
ELLIOTT, ALFRED M., AND ROBERT E. HAYES
Mating types in Tetrahymena ..... 269
GRAY, I. E.
The relation of body weight to body surface area in marine fishes ..... 285
GREEN, JAMES W., AND JOSEPH F. HOFFMAN
A study of isotonic solutions for the erythocytes of some marine teleosts and elasmobranchs ..... 289
GROSCH, DANIEL S., AND ROBERT L. SULLIVAN
The fate of radiophosphorus ingested by Habrobracon females ..... 296
ITO, TOSHIO
Studies on the integument of the silkworm, Bombyx mori. VII. The permeability of the integument to oxygen and carbon dioxide in vivo .. 308
LASKER, REUBEN, AND CHARLES E. LANE
The origin and distribution of nitrogen in Teredo bartschi Clapp ..... 316
SCHNEIDERMAN, HOWARD A., AND CARROLL M. WILLIAMS
The physiology of insect diapause. VII. The respiratory metabolism of the Cecropia silkworm during diapause and development ..... 320
SMITH, RALPH I.
The distribution of the polychaete Neanthes lighti in the Salinas River estuary, California, in relation to salinity, 1948–1952 ..... 335
TAKATA, KENZO
Quantitative study on the regional distribution of pentose nucleic acid in the gastrula and neurula of Triturus ..... 348
Abstracts of papers presented at the Marine Biological Laboratory:
Tuesday evening seminars ..... 354
General Meetings ..... 361
Lalor Fellowship reports ..... 389

No. 3. DECEMBER, 1953

BLUM, JOHN L., AND JOHN T. CONOVER
New or noteworthy Vaucheriae from New England salt marshes ..... 395
BUCK, JOHN B., AND MARGARET L. KEISTER
Cutaneous and tracheal respiration in the Phormia larva ..... 402

PAGE

COMITA, JEAN J., AND ARTHUR H. WHITELEY
The correlation of desoxyribonucleic acid synthesis and the rate of respiration in the sea urchin embryo ..... 412

COSTLOW, JOHN D., JR., AND C. G. BOOKHOUT
Moulting and growth in Balanus improvisus ..... 420

FARNER, DONALD S., L. R. MEWALDT AND STANLEY D. IRVING
The roles of darkness and light in the photoperiodic response of the testes of white-crowned sparrows ..... 434

FRAENKEL, G., AND VICTOR J. BROOKES
The process by which the puparia of many species of flies become fixed to a substrate ..... 442

GOLDMAN, ALLEN S.
Synthesis of pigment during the reconstitution of Tubularia ..... 450

GREGG, JOHN R., AND NORMA ORNSTEIN
Explant systems and the reactions of gastrulating amphibians to metabolic poisons ..... 466

JØRGENSEN, C. BARKER, AND EDWARD D. GOLDBERG
Particle filtration in some ascidians and lamellibranchs ..... 477

LEHMAN, H. E.
Observations on macrophage behavior in the fin of Xenopus larvae .... 490

---

## ERRATUM

On page 162 of Volume 105 (August, 1953 issue) in the article by H. L. Rosenthal, the first two sentences of the final paragraph should read as follows:

The data presented in Table II show the changes that occur in the nitrogen, calcium and phosphorus content of the dry fat-free tissue during growth and development of the Lebistes embryo. It is apparent that the total protein remains essentially constant for both the lordotic and normal strains although the values obtained for the lordotic strain are somewhat smaller due to the smaller size of the embryo.

BIO

Volume 105 Number 1

UNIVERSITY
OF MICHIGAN
SEP 0 1953
SCIENCE
LIBRARY

# THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

Editorial Board

W. C. ALLEE, University of Florida
L. R. BLINKS, Stanford University
L. V. HEILBRUNN, University of Pennsylvania
A. K. PARPART, Princeton University
BERTA SCHARRER, University of Colorado
F. SCHRADER, Columbia University
H. B. STEINBACH, University of Minnesota
WM. RANDOLPH TAYLOR, University of Michigan
ALBERT TYLER, California Institute of Technology
JOHN H. WELSH, Harvard University
DOUGLAS WHITAKER, Stanford University
RALPH WICHTERMAN, Temple University

DONALD P. COSTELLO, University of North Carolina
*Managing Editor*

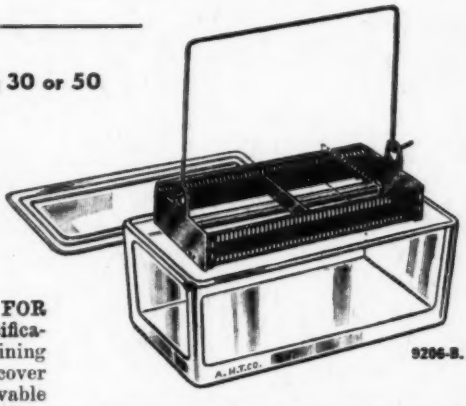
AUGUST, 1953

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

A.H.T. CO. SPECIFICATION

# STAINING ASSEMBLIES for MICRO SLIDES

- **Nickel silver Racks for holding 30 or 50 slides in vertical position**
- **NEW, private mould, heavy wall, carefully finished Glass Dishes**




9206-B.

**STAINING ASSEMBLIES FOR MICRO SLIDES, A.H.T. Co. Specification.** Consisting of a glass Staining Dish with loosely fitting flat glass cover which permits stacking, and removable Rack of nickel silver which takes slides in horizontal position in individual slots.

The Dishes have been specially designed for use with these Racks and are now made in new private moulds, with heavy walls and slightly tapering sides, carefully finished throughout. Outside dimensions of the Dish for thirty slides are 5 inches long × 4 inches wide at top × 3 inches high; outside dimensions of the Dish for 50 slides are 8¼ inches long × 4½ inches wide at top × 3¼ inches high. Inside dimensions of both dishes accommodate 3 × 1, 3 × 1½, or 3 × 2-inch slides with economical use of staining solutions.

The Racks are of nickel silver, with solderless joints, and are resistant to the action of most of the reagents used in staining techniques.

| | |
|---|---|
| 9203-B. | **Staining Assembly,** as above described, consisting of metal Rack for 30 slides, Glass Dish with flat glass cover, and removable wire handle for the Rack..2.98 |
| 9204. | **Staining Rack,** only, of nickel silver, for thirty slides, without wire handle .....1.25 |
| 9205. | **Glass Dish,** only, with loosely fitting glass cover .....1.45 |
| 9206-B. | **Staining Assembly,** as above described, consisting of metal Rack for fifty slides, Glass Dish with flat glass cover, and removable wire handle for the Rack.....3.68 |
| 9207. | **Staining Rack,** only, of nickel silver, for fifty slides, without wire handle .....1.70 |
| 9208. | **Glass Dish,** only, with loosely fitting glass cover .....1.65 |


## ARTHUR H. THOMAS COMPANY

*Laboratory Apparatus and Reagents*

WEST WASHINGTON SQUARE PHILADELPHIA 5, PA.

Teletype Services: Western Union WUX and Bell System PH-72

Vol. 105, No. 1 August, 1953

# THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

## THE MARINE BIOLOGICAL LABORATORY

FIFTY-FIFTH REPORT, FOR THE YEAR 1952—SIXTY-FIFTH YEAR


I. TRUSTEES AND EXECUTIVE COMMITTEE (AS OF AUGUST 14, 1952) ..... 1
STANDING COMMITTEES
II. ACT OF INCORPORATION ..... 4
III. BY-LAWS OF THE CORPORATION ..... 4
IV. REPORT OF THE TREASURER ..... 6
V. REPORT OF THE LIBRARIAN ..... 10
VI. REPORT OF THE DIRECTOR ..... 11
Statement ..... 11
Addenda:
1. Memorial ..... 14
2. The Staff ..... 16
3. Investigators and Students ..... 19
4. The Lalor Fellows ..... 26
5. Tabular View of Attendance, 1948–1952 ..... 27
6. Subscribing and Cooperating Institutions ..... 27
7. Evening Lectures ..... 28
8. Shorter Scientific Papers (Seminars) ..... 29
9. Members of the Corporation ..... 30


## I. TRUSTEES

EX OFFICIO

GERARD SWOPE, JR., *President of the Corporation*, 570 Lexington Ave., New York City
E. NEWTON HARVEY, *Vice President of the Corporation*, Princeton University
PHILIP B. ARMSTRONG, *Director*, State University of New York, Medical Center at Syracuse
C. LLOYD CLAFF, *Clerk of the Corporation*, Randolph, Mass.
JAMES H. WICKERSHAM, *Treasurer*, 530 Fifth Ave., New York City

EMERITI

EUGENE DUBOIS, Cornell University Medical College
G. H. A. CLOWES, Lilly Research Laboratory

E. G. CONKLIN, Princeton University
ROBERT CHAMBERS, Dade County Cancer Institute
W. C. CURTIS, University of Missouri
B. M. DUGGAR, University of Wisconsin
ROSS G. HARRISON, Yale University
F. P. KNOWLTON, Syracuse University
A. P. MATHEWS, University of Cincinnati
W. J. V. OSTERHOUT, Rockefeller Institute
G. H. PARKER, Harvard University

TO SERVE UNTIL 1956

W. C. ALLEE, University of Florida
E. S. G. BARRON, University of Chicago
D. W. BRONK, Johns Hopkins University
G. FAILLA, College of Physicians and Surgeons
L. V. HEILBRUNN, University of Pennsylvania
R. T. KEMPTON, Vassar College
A. H. STURTEVANT, California Institute of Technology
GEORGE WALD, Harvard University

TO SERVE UNTIL 1955

C. L. BURDICK, The Lalor Foundation, Wilmington, Delaware
E. G. BUTLER, Princeton University
K. S. COLE, Naval Medical Research Institute
H. HIBBARD, Oberlin College
M. H. JACOBS, University of Pennsylvania, School of Medicine
H. A. MOE, Guggenheim Memorial Foundation
J. S. RANKIN, University of Connecticut
R. RUGH, Radiological Research Laboratory, College of Physicians & Surgeons

TO SERVE UNTIL 1954

A. M. CHASE, Princeton University
D. P. COSTELLO, University of North Carolina
ETHEL B. HARVEY, Princeton University
D. A. MARSLAND, New York University
D. MAZIA, University of California
C. PACKARD, Woods Hole
A. K. PARPART, Princeton University
H. B. STEINBACH, University of Minnesota

TO SERVE UNTIL 1953

W. R. AMBERSON, University of Maryland School of Medicine
E. G. BALL, Harvard University Medical School
F. A. BROWN, JR., Northwestern University
ROBERT CHAMBERS, New York University
LEWIS KLEINHOLZ, Reed College
C. L. PROSSER, University of Illinois
A. C. REDFIELD, Harvard University and Woods Hole Oceanographic Institution
ALBERT TYLER, California Institute of Technology

### Executive Committee of the Board of Trustees

Gerard Swope, Jr., *Chairman*
E. N. Harvey, *Ex officio*
James H. Wickersham
P. B. Armstrong, *Ex officio*
E. G. Ball
E. G. Butler
F. A. Brown, Jr., to serve until 1953
D. P. Costello, to serve until 1953
A. K. Parpart, to serve until 1954
H. B. Steinbach, to serve until 1954

### The Library Committee

W. R. Taylor, *Chairman*
H. W. Stunkard
E. G. Butler
Mary Sears
H. Blum

### The Apparatus Committee

H. B. Steinbach, *Chairman*
Ivor Cornman
A. M. Chase
O. H. Schmitt
C. L. Prosser
D. Mazia

### The Supply Department Committee

P. B. Armstrong, *Chairman*
C. L. Claff
C. B. Metz
L. H. Kleinholz

### The Evening Lecture Committee

P. B. Armstrong, *Chairman*
E. G. Ball
E. G. Butler
G. H. A. Clowes
H. H. Plough

### The Instruction Committee

F. A. Brown, Jr., *Chairman*
Hope Hibbard
W. R. Taylor
D. P. Costello

### The Buildings and Grounds Committee

C. Lloyd Claff, *Chairman*
Ralph Wichterman

## II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

*Now, therefore*, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

*Witness* my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
*Secretary of the Commonwealth.*

---

## III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:00 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time

and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 9:00 A.M. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. Any person interested in the Laboratory may be elected by the Trustees to a group to be known as Associates of the Marine Biological Laboratory.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

## IV. REPORT OF THE TREASURER

The Treasurer's Report for 1952 is this year presented in a slightly different form. A condensation of the Auditor's Income and Expense statements for the past five years is submitted, which I hope you will all study carefully. It points out the need of the Laboratory for additional income and the importance of continued efforts to be put forth to secure special donations each year, as it is obvious that it is these special donations which have permitted the Laboratory to continue functioning.

In addition, the Balance Sheet, Condensed Income and Expense Statements and Statement of Surplus as reported in the annual audit by the firm of Seamans, Stetson and Tuttle of Boston are included, as well as a summary of the cash transactions passing through our various banking accounts.

There has been a little change in the market value of the securities in the Endowment Funds during the past year. The market value on January 1, 1953, was $1,166,063.74 in comparison to the market value shown January 1, 1952 of $1,108,-645.75. The average yield on the securities was 4.55% on book value, 3.78% on market value. The investments and percentages in the Endowment Funds as of January 1, 1953, were:

| | Book Value | % | Market Value | % |
|---|---|---|---|---|
| *Bonds* | | | | |
| U. S. Government | $344,004.81 | 35.45 | $ 333,155.27 | 28.57 |
| Railroad | 54,613.02 | 5.63 | 58,545.00 | 5.02 |
| Public Utility | 90,192.50 | 9.29 | 84,737.50 | 7.27 |
| Industrial | 61,518.75 | 6.34 | 60.687.50 | 5.20 |
| Total Bonds | 550,329.08 | 56.71 | 537,125.27 | 46.06 |
| *Preferred Stocks* | 127,173.50 | 13.11 | 120,632.75 | 10.35 |
| *Common Stocks* | 290.104.71 | 29.89 | 505,494.38 | 43.35 |
| Total Securities | 967,607.29 | | 1,163,252.40 | |
| Principal Cash | 2,811.34 | .29 | 2,811.34 | .24 |
| *Totals* | $970,418.63 | 100. | $1,166,063.74 | 100. |

### MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1952

*Assets*

| | | |
|---|---|---|
| *Endowment Assets and Equities:* | | |
| Securities and Cash in Hands of the Hanover Bank, New York, Trustee | $ 970,418.63 | |
| Securities and Cash in Minor Funds | 21,198.88 | $ 991,617.51 |
| *Plant Assets:* | | |
| Land | $ 113,626.38 | |
| Buildings | 1,488,930.19 | |
| Equipment | 333,012.06 | |
| Library | 431,834.91 | |
| | $2,367,403.54 | |
| Less Reserve for Depreciation | 850,842.36 | $1,516.561.18 |

| | | | |
|---|---|---|---|
| *Current Assets:* | | | |
| Cash | | $ 35,988.19 | |
| Mortgage Note Receivable | | 2,350.00 | |
| Accounts Receivable | | 18,462.80 | |
| Inventories: | | | |
| Supply Department | 57,123.96 | | |
| Biological Bulletin | 16,707.09 | 73,831.05 | |
| Investments: | | | |
| Devil's Lane Property | 36,881.56 | | |
| Stock in Gen. Biol. Supply House, Inc. | 12,700.00 | | |
| Other Investment Securities | 47,101.25 | | |
| Retirement Fund | 22,142.96 | 118,825.77 | |
| Prepaid Insurance | | 6,605.40 | |
| Items in Suspense (Debits) | | 686.80 | 256.750.01 |
| | | | $2,764,928.70 |

*Liabilities*

| | | | |
|---|---|---|---|
| *Endowment Funds:* | | | |
| Endowment Funds | 967,974.68 | | |
| Reserve for Amortization | 2,443.95 | 970,418.63 | |
| Minor Funds | | 21,198.88 | 991,617.51 |
| *Plant Funds:* | | | |
| Mortgage Note Payable | | 5,000.00 | |
| Donations and Gifts | 1,404,441.81 | | |
| Other Investments in Plant from Gifts and Current Funds | 107,119.37 | 1,511,561.18 | 1,516,561.18 |
| *Current Liabilities and Surplus:* | | | |
| Accounts Payable | | 31,473.84 | |
| Items in Suspense (Credits) | | 3,549.31 | |
| Current Surplus | | 221,726.86 | 256.750.01 |
| | | | $2,764,928.70 |

## MARINE BIOLOGICAL LABORATORY

*Five Year Condensed Income and Expense Statement*

Reference—Exhibit B Auditors' Reports—1948 through 1952

| | 1952 | 1951 | 1950 | 1949 | 1948 | Total |
|---|---|---|---|---|---|---|
| Income from Endowments | $ 44,115.00 | $ 44,898.43 | $ 46,431.59 | $ 40,347.15 | $ 37,586.48 | $ 213,378.65 |
| Income from Investments, Including Retirement Fund and Mortgage Rec. | 21,635.50 | 23,184.86 | 22,844.19 | 22,593.55 | 25,570.93 | 115,829.03 |
| Income from Fees and Dues, including Instruction, Research, Library | 46,695.76 | 46,633.00 | 47,888.61 | 44,921.33 | 40,321.04 | 226,459.74 |
| Income from Mess, Dormitories, Supply, Biol. Bulletin, Sundry—Net | 19,690.73 | 12,943.63 | 21,601.35 | 27,499.18 | 28,387.61 | 110,122.50 |
| Income from Rents, including Bar Neck | 7,333.26 | 8,047.42 | 7,090.89 | 6,330.00 | 6,360.00 | 35,161.57 |
| TOTAL ORDINARY INCOME....A | 139,470.25 | 135,707.34 | 145,856.63 | 141,691.21 | 138,226.06 | 700,951.49 |
| Income by Special Donations | 65,242.69 | 77,730.00 | 43,130.66 | 37,459.45 | 29,375.00 | 252,937.80 |
| TOTAL INCOME....X | $204,712.94 | $213,437.34 | $188,987.29 | $179,150.66 | $167,601.06 | $ 953,889.29 |
| Buildings, Grounds, and Other Property Expense | $ 51,020.70 | $ 56,353.84 | $ 53,066.36 | $ 41,591.16 | $ 39,536.68 | $ 241,568.74 |
| Research Service Expense—Apparatus and Chemical Rooms | 24,312.98 | 25,492.99 | 25,244.54 | 23,901.70 | 24,721.91 | 123,674.12 |
| Instruction, Research and Library Expense | 34,632.76 | 34,083.50 | 34,077.76 | 30,587.06 | 30,217.79 | 163,598.87 |
| General Expenses, Administration, Pension Fund, Bank Charges, etc. | 39,396.93 | 41,375.04 | 37,623.08 | 43,650.91 | 38,039.39 | 200,085.35 |
| | 149,363.37 | 157,305.37 | 150,011.74 | 139,730.83 | 132,515.77 | 728,927.08 |
| Depreciation | 34,359.93 | 32,181.00 | 29,160.29 | 27,919.94 | 26,666.86 | 150,288.02 |
| | 183,723.30 | 189,486.37 | 179,172.03 | 167,650.77 | 159,182.63 | 879,215.10 |
| Plant Additions from Current Funds | 19,360.99 | 16,767.59 | 17,775.06 | 33,408.40 | 49,321.22 | 136,633.26 |
| TOTAL ORDINARY EXPENSE....B | 203,084.29 | 206,253.96 | 196,947.09 | 201,059.17 | 208,503.85 | $1,015,848.36 |
| Special Expenditures from Donations | 10,433.11 | 9,705.88 | 5,129.49 | ..... | ..... | 25,268.48 |
| | 213,517.40 | 215,959.84 | 202,076.58 | 201,059.17 | 208,503.85 | 1,041,116.84 |
| Plant Additions from Donations | 23,722.13 | 44,020.64 | ..... | ..... | ..... | 67,742.77 |
| TOTAL EXPENSE....Y | $237,239.53 | $259,980.48 | $202,076.58 | $201,059.17 | $208,503.85 | $1,108,859.61 |
| TOTAL NET (X−Y) | **32,526.59** | **46,543.14** | **13,089.29** | **21,908.51** | **40,902.79** | **154,970.32** |
| Delete Depreciation | 34,359.93 | 32,181.00 | 29,160.29 | 27,919.94 | 26,666.86 | 150,288.02 |
| Total Net, less Depreciation | 1,833.34 | **14,362.14** | 16,071.00 | 6,011.43 | **14,235.93** | **4,682.30** |
| Operating Net (A−B) | **63,614.04** | **70,546.62** | **51,090.46** | **59,367.96** | **70,277.79** | **314,896.87** |
| Delete Depreciation | 34,359.93 | 32,181.00 | 29,160.29 | 27,919.94 | 26,666.86 | 150,288.02 |
| Operating Net, Less Depreciation | **$ 29,254.11** | **$ 38,365.62** | **$ 21,930.17** | **$ 31,448.02** | **$ 43,610.93** | **$ 164,608.85** |

*Marine Biological Laboratory Current Surplus December 31, 1952*

| | | | |
|---|---|---|---|
| Balance, January 1, 1952 | | | $217,152.46 |
| *Add:* | | | |
| Excess of Income over Expense for Year | | $10,556.53 | |
| Prior year's adjustments, Net Recovery | | 2,741.06 | |
| Reserve for Depreciation, 1952, Charged to Plant Funds | | 34,359.93 | 47,657.52 |
| | | | 264,809.98 |
| *Deduct:* | | | |
| Payments during year for Plant Assets: | | | |
| Equipment: | | | |
| From Current Funds | $ 322.50 | | |
| From Rockefeller Foundation Grant for Special Apparatus | 20,711.19 | | |
| From Lillie Memorial Fund | 3,010.94 | 24,044.63 | |
| Library | | 13,038.49 | |
| | | 37,083.12 | |
| Mortgage Note Payment, Elliot House | | 6,000.00 | 43,083.12 |
| Balance, December 31, 1952 | | | $221,726.86 |

*Summary of Bank Balance*

| | | |
|---|---|---|
| Cash Balance January 1, 1952 | | |
| The Hanover Bank | $ 2,923.71 | |
| Falmouth National Bank | 930.25 | $ 3,853.96 |
| Receipts | | 373,097.64 |
| | | 376,951.60 |
| Payments | | 348,856.48 |
| Cash Balance, December 31, 1952 | | |
| The Hanover Bank | 25,547.59 | |
| Falmouth National Bank | 2,547.53 | 28,095.12 |

| Other Cash Accounts | Balances Jan. 1, 1952 | Received | Paid | Balances Dec. 31, 1952 |
|---|---|---|---|---|
| Allen R. Memhard Fund | $ 104.15 | $ 27.50 | $ 45.00 | $ 86.65 |
| Rev. Arsenious Boyer Fund | 298.82 | 148.76 | 270.00 | 177.58 |
| Lucretia Crocker Fund | 739.83 | 452.55 | | 1,192.38 |
| G. H. A. Clowes Beach Fund | 1,000.00 | | | 1,000.00 |
| F. R. Lillie Memorial Research Fund | 7,986.81 | 6,225.00 | 7,818.74 | 6,393.07 |
| Retirement Fund | 2,495.45 | | | |
| Bio Club Scholarship Fund | 127.24 | 873.76 | 206.25 | 794.75 |
| Lalor Foundation Fellowship Fund | 1,354.42 | 6,000.00 | 6,377.92 | 976.50 |

Respectfully submitted,
JAMES H. WICKERSHAM,
*Treasurer*

## V. REPORT OF THE LIBRARIAN

### 1952

The appropriation for 1952 Library expenses was $15,128 plus $4500 (to be applied to staff salaries) from the Woods Hole Oceanographic Institution. This sum was expended as follows: Serials, $5650.57; Books, $613.79; Back Sets, $74.51; Binding, $2351.90; Supplies, Sundries, Freight and Express, $411.49; Insurance, $55.00; and Salaries, $8328.25; making a total of $17,485.51.

The yearly allowance from the Woods Hole Oceanographic Institution, to acquire oceanographic books and journals and the binding of same, was $1200 plus a balance of $132.08 from 1951. Of this amount, $977.04 was expended, leaving a balance of $355.04 on December 31, 1952, which will be used for an outstanding back-set order.

The titles of current journals received in 1952 numbered 1479 (65 new). There were 450 (6 new) Marine Biological Laboratory subscriptions; 596 exchanges (24 new) and 164 gifts (12 new); 72 (4 new) were Woods Hole Oceanographic Institution subscriptions; 168 (13 new) were exchanges and 29 (6 new) were gifts.

The Marine Biological Laboratory purchased 55 books, received 82 complimentary copies (7 from authors and 75 from publishers) and 16 miscellaneous donations. The Woods Hole Oceanographic Institution purchased 29 titles and received 18 gifts. The total number of new books placed on the shelves was 200.

During World War II, there occurred hiatuses in the current receipts of foreign periodicals. In 1952, four complete volumes and 50 odd numbers were secured, greatly lowering the number of missing items. In a few years, it is hoped that all gaps will be filled in, either in the original or in photographic copy. The Woods Hole Oceanographic Institution partially completed four back sets by purchase and one complete set and one partially complete set were received as gifts.

The reprint collection was increased by 4146 papers, 2032 being of current issue and the remaining ones of earlier dates.

Ninety-three titles were borrowed on inter-library loan and 111 were sent to out-of-town institutions. There were 46 microfilm orders filled, the service earning $273.91. (It was necessary to suspend the service the latter part of the year due to a change in the staff.) A sum of $211.28 was realized from the sale of duplicate material.

The second edition of the list of journal holdings was published in the February issue of the BIOLOGICAL BULLETIN. There are 2680 titles with holdings listed, 1432 of these being of current issue.

In 1952, the Library had the great fortune of receiving the remainder of Dr. Rudolph Höber's collection of reprints on physiological chemistry. The mass of his collection was presented to the Laboratory in 1942. This valuable gift totalled 12,000 papers. In appreciation, a bronze plaque with appropriate inscription was placed in the Library.

At the end of the year 1952, the Library contained 62,151 bound volumes and 182,941 reprints.

For the helpfulness and advice so willingly contributed by the members of the Library Committee, the Librarian wishes to express grateful thanks and appreciation.

Respectfully submitted,
DEBORAH L. HARLOW,
*Librarian*

---

## VI. REPORT OF THE DIRECTOR

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

Gentlemen:

I submit herewith a report of the sixty-fifth session of the Marine Biological Laboratory.

The Laboratory continues its progress in the development of its various services. There was added to our equipment this year an electron microscope purchased from the equipment grant of $75,000 made to the Laboratory by the Rockefeller Foundation. Use of the instrument was supervised by Dr. James Hillier. It was in almost continuous daily operation. The total number of scientific services now rendered by the Service Department number 9 which, in addition to the electron microscope, include the radioisotopes laboratory service, X-ray service, special apparatus service (ultracentrifuge, preparation centrifuge, refrigerated centrifuge, freezing-drying apparatus, electrophoresis apparatus, etc.), apparatus supply and repair service, chemistry supply service, glassblowing, carpenter shop service, and biological collecting and supply service.

*1. Changes in Officers:*

This year was marked by the retirement of Mr. Lawrason Riggs as President of the Corporation. Mr. Riggs served the Laboratory faithfully and ably as Treasurer from 1924 to 1942. In 1942 he succeeded Dr. Frank R. Lillie as President of the Corporation. Mr. Riggs served during the trying war years when the problems of the Laboratory were most difficult. The period since then has been marked by a physical rehabilitation of the Laboratory, many plant improvements including a complete rebuilding of Old Main, marked expansion of our research equipment and services and improvement of our biological collecting facilities. The Laboratory was fortunate in having such able leadership during this critical period.

Mr. Riggs was succeeded by Mr. Gerard Swope, Jr., Vice-President of the General Electric Company, who brings to the position deep interest and great enthusiasm.

Dr. E. Newton Harvey retired at the end of the year as Vice-President of the Corporation. It was with great reluctance that the Trustees acceded to Dr. Harvey's wish to retire. His broad scientific knowledge and excellent judgment were invaluable during his 11 year term as Vice President.

Dr. Arthur K. Parpart, who has been in regular attendance at the Laboratory since 1924 and who is so well-versed in its problems, was elected to succeed Dr. Harvey.

Mr. Donald M. Brodie who, since 1942, has served the Laboratory as Treasurer of the Corporation, also requested retirement. The Laboratory is grateful for his years of service and his wise counsel in our financial affairs.

Mr. James H. Wickersham, Vice-President of the Bank of New York, succeeded Mr. Brodie and here again the Laboratory was fortunate in acquiring the support and assistance of a man with broad experience to advise us.

*2. Instruction:*

The course in Marine Ecology in its initial year operated most effectively. A large number of students and investigators from the three Woods Hole Institutions monitored the course in which there was a sustained interest. The staff under the direction of Dr. Bostwick H. Ketchum is to be commended on the results they achieved. The New York Zoological Society has renewed its grant of $4,000 for another year's support of this course.

*3. Increases in Rates:*

When the Executive Committee met at the end of the year to set up the budget for 1953, it was immediately obvious that there would be a sizable deficit unless new funds were obtained. The salaries paid the full time staff had lagged considerably behind the increased costs of living since the war so that increases were essential. A part of this will be met by an average 20% increase in the laboratory rental space. Current negotiations with the National Science Foundation and the Office of Naval Research may provide additional funds through contracts which will help cover the prospective deficit.

*4. The Bar Neck Property:*

In November the Government instituted condemnation proceedings for the taking of the Bar Neck Property as the site for its new Oceanographic Laboratory. The M. B. L. could fight condemnation; however, this would be very expensive and fruitless according to legal advice obtained by the Executive Committee. During the war the Armed Services were given unusual powers of condemnation which have not yet been rescinded. The loss of this property is a serious blow to the Laboratory, both income-wise and in terms of its future development. The Bar Neck site appears to have been chiefly a matter of convenience rather than one of necessity since there were other locations on which the Government could have put its new laboratory. Also, it appears that The Marine Biological Laboratory will be unable to recover adequate damages for the loss of the property since some of the features which made it so valuable to the Laboratory probably will not be considered pertinent in an appraisal.

*5. Grants, Contributions and Gifts:*

These are assuming great importance in the support of the Laboratory. This past year they totaled $65,242.69.

Grants:

| | |
|---|---|
| Rockefeller Foundation, general support | $17,500 |
| Rockefeller Foundation, special apparatus (balance) | 25,000 |
| American Cancer Society, radioisotopes laboratory | 1,000 |
| Kettering Foundation, general support | 2,500 |
| American Philosophical Society, laboratory support | 1,800 |
| New York Zoological Society, ecology course | 4,000 |

Contributions:

| | |
|---|---|
| Eli Lilly Company | 5,000 |
| Mr. Lawrason Riggs | 1,000 |
| Dr. Charles Kettering | 2,500 |
| Mrs. W. Murray Crane | 1,300 |
| Dr. W. D. Curtis | 463 |

Gifts:

Mr. H. D. Loring has presented to the Laboratory a new 25-foot power boat which will be an important addition to the fleet of boats used by the Supply Department for the collection of biological materials. The name chosen for the boat, "Loligo," is in the Laboratory tradition, the name of a marine form used extensively in research.

The generosity of the various supporters of the Laboratory is deeply appreciated by the Corporation of the Marine Biological Laboratory. It is also a most gratifying expression of their faith in the Laboratory.

*6. M. B. L. Associates:*

The Associates of the Laboratory now number 95. They contributed $1610 to the Laboratory this past year, which has been ear-marked for the reconstruction of the marine railway. A part of this work will be carried out before the summer of 1953.

*7. Deaths:*

The Corporation suffered the loss this year of one of its most distinguished and oldest members with the death of Dr. Edwin Grant Conklin who played such an important role in the development of the Laboratory during its formative years. His researches were also among the most notable coming from the Laboratory.

Respectfully submitted,
PHILIP B. ARMSTRONG,
*Director*

## 1. Memorial

*Ralph Stayner Lillie*

1875–1952

By

Dr. E. G. Conklin

In the death of Ralph Lillie on March 19, 1952, the Marine Biological Laboratory lost one of its early and most constant members and the world of science and philosophy a scholar of distinction. He was born in Toronto, Canada, August 8, 1875, about five years after the birth of his brother, Frank R. Lillie, and his education, culminating in the degree A.B. from the University of Toronto in 1896 and of Ph.D. from the University of Chicago in 1901, followed by five years the same degrees from the same universities conferred upon his brother Frank. In spite of these similarities in their origin and education Ralph Lillie was from the first a scientist and scholar of marked individuality; his chosen field of work was general physiology and its relations to the great problems of psychology and philosophy, rather than the prevailing work in morphology and morphogenesis, which characterized so much of the research of this Laboratory in the early years of this century.

After receiving the doctor's degree at the University of Chicago in 1901, he was an assistant in physiology at Harvard for one year, and then instructor and adjunct professor of physiology at the University of Nebraska for three years; in 1904–05 he was appointed by the Carnegie Institution of Washington, as one of its research assistants to occupy a table in the Naples Zoological Station, and this was followed by one year as instructor in physiology at Harvard and by another as Johnston Scholar at Johns Hopkins University.

At that time physiology was generally regarded as a strictly medical school subject, with emphasis on the physiology of higher animals and men, and with small place for such general physiology as Ralph Lillie was committed to, but in 1907 he was appointed, under my selection, assistant professor of general physiology and experimental zoology in the Department of Zoology at the University of Pennsylvania. He continued in that position until 1913 when he was called to be professor of biology in Clark University. In 1920 he was appointed research biologist in the laboratory of the National Electric Light Association in Cleveland, and after four years in that position he finally came in 1924 to the professorship of general physiology in the University of Chicago, which position he held with honor and distinction until his retirement for age in 1940, when he became professor emeritus.

Dr. Lillie was a thinker and writer rather than a public speaker and while he was a member of most of the national societies of biology, physiology, and philosophy, including the two oldest and most distinguished learned societies of America, he rarely attended their meetings or took part in their programs, owing in part to a slight defect in hearing. One must, therefore, depend largely on his publications in estimating the character and extent of his work. In the library of the Marine Biological Laboratory there are 120 reprints of his research papers and two books, one of these in two editions, which are listed in the card catalogue under some 20 topics in general physiology, while his philosophical writings are grouped together under the topic "theoretical biology." About 96 of these reprints are primarily in general biology, while 24 are more largely philosophical. It is significant that he published four times as many papers in physiology as in philosophy

and that all of his papers from 1901 to 1914 were in physiology, while almost all in the last decade of his life were philosophical. In short, his philosophy was an outgrowth of his physiology and an attempt to fit the facts of biology—all facts of life including human life—into a logical and practical system.

Among his more important contributions to physiology should be mentioned the effects of various salt solutions on ciliary and muscular movement; relations of ions to contractility and irritability; influence of electrolytes on osmotic pressure of colloidal solutions; the connection between permeability of plasma membranes and irritability, contractility and living processes in general; and, growing out of this, some seven papers on the physiology of anaesthesia. Four main papers were devoted to the physiology of cell division in which he pointed out similarities between certain floating bodies in electrical fields and the positions of chromosomes in mitosis—a model which has been widely used in teaching cytology. Several papers were on the rate of conduction in nerve and irritable tissues, and in connection with this he invented the iron-wire model of such transmission, recovery and rhythmical activity, which is now widely used in explaining these phenomena. This is merely a brief mention of some of his more important and original contributions to general physiology.

In philosophy he was not content to leave out of account all phenomena not now explicable by current materialistic theories, or to explain them by explaining them away as non-existent. He maintained that man and all his psychical qualities must be included in any comprehensive philosophy of nature, for he is a part of nature. He defined his outlook on such a philosophy as one of "critical naturalism" and he held that present mechanistic science is based too largely on physics and static conditions, and takes too little account of biology, creative synthesis and novelty. He held that "every living organism is the psycho-physical unity," not a duality of soul and body, and in general he agreed with the philosophy of Alfred North Whitehead. It is difficult and indeed impossible in a brief summary, to do justice to his profound philosophy but it is summarized in his last book, General Biology and Philosophy of Organism (University of Chicago Press, 1945).

Throughout his whole professional life Ralph Lillie was intimately associated with the Marine Biological Laboratory. In the summer of 1896, immediately following his graduation from the University of Toronto, he was a graduate student in the embryology class here, and according to the Annual Reports of the Laboratory, and the memories of friends and associates, he was present as investigator or instructor for at least a part of every summer since 1896, a total of 55 sessions, which is almost a unique record. From 1902 to 1922 he was instructor in the class in comparative physiology, and thereafter until 1941 he was a member of the "staff of instruction in physiology," since which time he has been recorded as a member of the "senior staff of instruction." In 1921 he was elected a Trustee of the Laboratory for a term of four years and on five different occasions since then at the expiration of his term he was re-elected to the board; finally in 1945 he was elected trustee emeritus.

Not only did Ralph Lillie's research life have its center here at Woods Hole but some of his most intimate social and family relations also centered here. It is a notable fact that many, perhaps a majority of the earlier workers at the Laboratory, who were not already married, met their matrimonial mates here among students and co-workers and such marriages have been both life-long and eugenic; it is a record of which this Laboratory may well be proud. Here Ralph Lillie and Helen Makepeace met, became engaged, and in 1906 were married, and here they have ever since made their summer home, which has been blessed by two worthy sons, and which in turn has blessed many friends who have enjoyed their charming hospitality. Dr. Lillie was a pianist of note and the artistic and literary quality of their home added much to the cultural atmosphere of Woods Hole.

It is not surprising that those who have lived and worked here and have enjoyed the associations and pleasures of this place should love it beyond all others, nor that they should cherish the hope of leaving not only their thoughts and examples but also their mortal remains in this Valhalla of Biologists. This was the desire of Ralph Lillie and his family, and after a beautiful funeral service in the Church of the Messiah on April 3, 1952, his ashes were buried in the churchyard here where rest so many of our honored dead.

## 2. The Staff, 1951

Philip B. Armstrong, Director, State University of New York, School of Medicine, Syracuse.

### Senior Staff of Investigation

E. G. Conklin, Professor of Zoology, *Emeritus,* Princeton University.
A. P. Mathews, Professor of Biochemistry, *Emeritus,* University of Cincinnati.
G. H. Parker, Professor of Zoology, *Emeritus,* Harvard University.

### ZOOLOGY

#### I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University.
Libbie H. Hyman, American Museum of Natural History.
A. C. Redfield, Woods Hole Oceanographic Institution.

#### II. Instructors

L. H. Kleinholz, Associate Professor of Biology, Reed College, in charge of course.
George M. Moore, Professor of Zoology, University of New Hampshire.
C. G. Goodchild, Professor of Biology, S. W. Missouri State College.
John H. Lochhead, Associate Professor of Zoology, University of Vermont.
Madelene E. Pierce, Associate Professor of Zoology, Vassar College.
Marion H. Pettibone, Arctic Research Laboratory, U. S. National Museum.
Nathan W. Riser, Department of Biology, Fisk University.
Joan C. Rattenbury, Department of Zoology, McGill University.

#### III. Laboratory Assistants

Robert S. Howard, Northwestern University.
Elizabeth Paulsen, Rutgers University.

### EMBRYOLOGY

#### I. Instructors

S. Meryl Rose, Associate Professor of Zoology, University of Illinois, in charge of course.
Charles B. Metz, Assistant Professor of Zoology, Yale University.
John T. Bonner, Assistant Professor of Biology, Princeton University.
Mac V. Edds, Jr., Associate Professor of Biology, Brown University.
John R. Shaver, Assistant Professor of Zoology, University of Missouri.
Edgar Zwilling, Associate Professor of Genetics, University of Connecticut.

### II. Laboratory Assistants

Lillian M. Youngs, University of North Carolina.
Joseph G. Gall, Yale University.

## PHYSIOLOGY

### I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania.
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine.
Arthur K. Parpart, Professor of Biology, Princeton University.
Albert Szent-Gyorgyi, Director, Institute for Muscle Research.

### II. Instructors

Daniel Mazia, Associate Professor of Zoology, University of California.
Michael Doudoroff, Associate Professor of Bacteriology, University of California.
Irving M. Klotz, Professor of Chemistry, Northwestern University.
Stephen Kuffler, Associate Professor, Johns Hopkins University.
H. Burr Steinbach, Professor of Zoology, University of Minnesota.
George Wald, Professor of Biology, Harvard University.
Milton Levy, Associate Professor of Chemistry, New York University College of Medicine.
Robert C. Warner, Assistant Professor of Chemistry, New York University College of Medicine.

### III. Laboratory Assistant

Paul Bernstein, Washington University.

## BOTANY

### I. Consultants

Maxwell S. Doty, Associate Professor of Botany, University of Hawaii.
Wm. Randolph Taylor, Professor of Botany, University of Michigan.

### II. Instructors

Francis T. Haxo, Assistant Professor of Biology, Johns Hopkins University, in charge of course.
Paul C. Silva, Department of Botany, University of California.
Richard C. Starr, Instructor in Botany, University of Indiana.

### III. Laboratory Assistant

Phyllis M. Strout, Yale University.

### IV. Collector

Peter Jennings, Drew University.

### V. Lecturers

J. B. Lackey, Medical and Scientific Editor, The Blakiston Company.
John Wallace, Academy of Natural Sciences of Philadelphia.

## MARINE ECOLOGY

### I. *Consultants*

W. C. Allee, University of Florida.
Alfred C. Redfield, Woods Hole Oceanographic Institution.

### II. *Instructors*

Bostwick H. Ketchum, Marine Microbiologist, Woods Hole Oceanographic Institution, in charge of course.
Edwin T. Moul, Assistant Professor of Botany, Rutgers University.
Charles Jenner, Assistant Professor of Zoology, University of North Carolina.

## EXPERIMENTAL RADIOLOGY

G. Failla, College of Physicians and Surgeons, Columbia University.
L. Robinson Hyde, Phillips Exeter Academy, Exeter, N. H.
Mones Berman, Memorial Cancer Center, New York City.

## LIBRARY

Mrs. Deborah Lawrence Harlow, Librarian
Mary A. Rohan

## APPARATUS DEPARTMENT

Robert Mills, Manager

James D. Graham — Betty J. Keeler — Seaver R. Harlow

## CHEMICAL DEPARTMENT

Gail M. Cavanaugh, Chemical Supplies Manager

## SUPPLY DEPARTMENT

James McInnis, Manager
John S. Rankin, Naturalist

Ruth Crowell — Patricia M. Conway

| | | |
|---|---|---|
| M. B. Gray | G. Lehy | James Whitcomb |
| A. M. Hilton | Carl O. Schweidenback | H. S. Wagstaff |
| W. E. Kahler | R. O. Lehy | Robert Perry |

## GENERAL OFFICE

Homer P. Smith, General Manager
Polly L. Crowell, Office Manager

Mrs. Lila S. Myers — Nancy Shave

## GENERAL MAINTENANCE

R. W. Kahler, Superintendent

| | |
|---|---|
| Robert Adams | James S. Thayer |
| Richard Alberts | Albert Neal |
| Robert Gunning | Alton J. Pierce |
| John H. Head | T. E. Tawell |

George A. Kahler

## 3. Investigators and Students

### Independent Investigators, 1952

Allee, W. C., Professor of Biology, University of Florida.
Allen, M. Jean, Assistant Professor of Zoology, University of New Hampshire.
Alscher, Ruth P., Assistant Professor of Biology, Manhattanville College of the Sacred Heart.
Anderson, Rubert S., Professor of Physiology, University of South Dakota.
Armstrong, Philip B., Professor of Anatomy, State University of New York at Syracuse.
Ball, Eric G., Professor of Biological Chemistry, Harvard University Medical School.
Bang, Frederick B., Associate Professor of Medicine, Johns Hopkins Hospital.
Bartlett, James H., Professor of Physics, University of Illinois.
Barton, Jay, II, Instructor in Zoology, Columbia University.
Berger, Charles A., Director, Biological Laboratory, Fordham University.
Blum, Harold F., Physiologist, NCI, and Visiting Lecturer, Princeton University.
Boettiger, Edward G., Assistant Professor of Zoology, University of Connecticut.
Bonner, John T., Associate Professor of Biology, Princeton University.
Boyce, Steve G., Teaching Fellow, North Carolina State College.
Boyer, Paul D., Associate Professor Ag. Biochemistry, University of Minnesota.
Bronk, Detlev, President, Johns Hopkins University.
Brown, Frank A., Jr., Professor and Chairman Biological Sciences, Northwestern University.
Butler, E. G., Professor of Zoology, Princeton University.
Chase, Aurin M., Associate Professor of Biology, Princeton University.
Cheney, Ralph H., Professor of Biology, Brooklyn College.
Ciereszko, Leon S., Assistant Professor of Chemistry, University of Oklahoma.
Claff, C. Lloyd, Research Associate in Surgery, Harvard Medical School.
Clark, Arnold M., Associate Professor of Biology, University of Delaware.
Clark, Eliot R., Professor of Anatomy, *Emeritus*, University of Pennsylvania School of Medicine.
Clement, A. C., Associate Professor of Biology, Emory University.
Clowes, G. H. A., Research Director, *Emeritus*, Eli Lilly and Company.
Cohen, Seymour S., Associate Professor, Childrens Hospital, University of Pennsylvania.
Cole, Kenneth S., Technical Director, Naval Medical Research Institute.
Colwin, Arthur L., Associate Professor of Biology, Queens College.
Connelly, Clarence M., Assistant Professor of Biophysics, Johns Hopkins University.
Cooperstein, Sherwin J., Senior Instructor in Anatomy, Western Reserve University.
Copley, Alfred L., Assistant Clinical Professor, New York Medical College.
Cornman, Ivor, Assistant Research Professor in Anatomy, George Washington University.
Costello, Donald P., Kenan Professor of Zoology, University of North Carolina.
Cranefield, Paul F., Post-doctorate Fellow, Johns Hopkins University.
Crowell, Sears, Assistant Professor of Zoology, Indiana University.
Csapo, Arpad I., Staff Member and Lecturer, Carnegie Institution of Washington.
Curtis, W. C., Professor of Zoology, *Emeritus*, University of Missouri.
DiStefano, Henry S., Instructor in Anatomy, State University of New York at Syracuse.
Doudoroff, Michael, Associate Professor of Bacteriology, University of California.
DuBois, Eugene F., *Emeritus* Professor of Physiology, Cornell University Medical College.
Edds, M. V., Jr., Associate Professor of Biology, Brown University.
Eichel, Bertram, Assistant Research Specialist, Rutgers University.
Eichel, Herbert J., Research Associate, Hahnemann Medical College.
Elliott, Alfred M., Associate Professor of Zoology, University of Michigan.
Failla, G., Professor of Radiology, Columbia University.
Figge, Frank H. J., Professor of Anatomy, University of Maryland Medical School.
Florkin, Marcel, Professor, University of Liège, Belgium.
Fraenkel, Gottfried, Professor of Entomology, University of Illinois.
Freeman, Paul J., Instructor in Physiology, San Jose State College.
Freygang, Walter, Jr., National Institute of Health.
Gasteiger, Edgar L., Research Fellow, Massachusetts General Hospital.

GILMAN, LAUREN C., Associate Professor of Zoology, University of Miami.
GOODCHILD, CHAUNCEY G., Professor of Biology, S. W. Missouri State College.
GOODRICH, HUBERT B., Professor of Biology, Wesleyan University.
GRANT, PHILIP, Public Health Fellow, Columbia University.
GRIFFIN, DONALD R., Associate Professor of Zoology, Cornell University.
GROSCH, DANIEL S., Associate Professor, Biological Division, North Carolina State College.
GRUNDFEST, HARRY, Associate Professor of Neurology, College of Physicians and Surgeons.
GUDERNATSCH, FREDERICK, 41 Fifth Avenue, New York 3, New York.
GUTTMAN, RITA, Assistant Professor, Brooklyn College.
HARVEY, ETHEL BROWNE, Independent Research Investigator, Princeton University.
HARVEY, E. NEWTON, Professor of Physiology, Princeton University.
HAXO, FRANCIS, Assistant Professor of Biology, Johns Hopkins University.
HAYASHI, TERU, Assistant Professor of Zoology, Columbia University.
HAYWOOD, CHARLOTTE, Professor of Physiology, Mount Holyoke College.
HEILBRUNN, L. V., Professor of Zoology, University of Pennsylvania.
HENDLEY, CHARLES D., Assistant Professor of Experimental Neurology, Tulane University.
HENLEY, CATHERINE, Research Associate, University of North Carolina.
HIBBARD, HOPE, Professor of Zoology, Oberlin College.
INOUÉ, SHINYA, Instructor in Anatomy, University of Washington, School of Medicine.
JACOB, MIRIAM I., Teaching Assistant, New York University.
JACOBS, MERKEL H., Professor of General Physiology, University of Pennsylvania.
JENKINS, GEORGE B., Professor of Anatomy, *Emeritus*, George Washington University.
JENNER, CHARLES E., Assistant Professor of Zoology, University of North Carolina.
KAO, CHIEN-JUAN, Assistant in Pathology, Cornell University Medical School.
KAVANAU, J. LEE, Research Fellow, University of California.
KEMPTON, RUDOLF T., Professor of Zoology, Vassar College.
KEOSIAN, JOHN, Professor of Biology, Rutgers University.
KIND, CHARLES ALBERT, Assistant Professor of Chemistry, University of Connecticut.
KING, JOHN W., Professor of Biology, Morgan State College.
KLEINHOLZ, L. H., Professor of Biology, Reed College.
KRAHL, MAURICE E., Associate Professor Biological Chemistry, Washington University School of Medicine.
KUFFLER, STEPHEN W., Associate Professor, Johns Hopkins University.
KUNTZ, ELOISE, Instructor in Physiology, Vassar College.
LANSING, ALBERT I., Associate Professor of Anatomy, Washington University School of Medicine.
LAZAROW, ARNOLD, Associate Professor, Western Reserve University.
LEVY, MILTON, Associate Professor, New York University College of Medicine.
LING, GILBERT, Instructor in Physiological Optics, Wilmer Institute, Johns Hopkins Hospital.
LOCHHEAD, JOHN H., Professor of Zoology, University of Vermont.
LOVE, WARNER E., Research Fellow, University of Pennsylvania.
LYNCH, WILLIAM F., Professor of Biology, St. Ambrose College.
MAKINO, SAJIRO, Professor of Zoology, Hokkaido University, Sapporo, Japan.
MARSLAND, DOUGLAS, Professor of Biology, Washington Square College, New York University.
MANKOWSKI, ZBIGNIEW T., Research Fellow, Cancer Institute.
MARSHAK, ALFRED, 32 Orange Street, Brooklyn 2, New York.
MAZIA, DANIEL, Associate Professor of Zoology, University of California.
MENKIN, VALY, Associate Professor of Experimental Pathology, Temple University School of Medicine.
METZ, CHARLES B., Assistant Professor of Zoology, Yale University.
MOORE, JOHN W., Biophysicist, Naval Medical Research Institute.
MOUL, EDWIN T., Assistant Professor of Botany, Rutgers University.
MULLAHY, JOHN H., Assistant Professor of Biology, Loyola University.
MUSACCHIA, X. J., Assistant Professor, Biological Labs., St. Louis University.
NACHMANSOHN, DAVID, Associate Professor of Neurology, Columbia University.
NEWTON, L. M., A. A. U. W. Fellow, British Museum (Natural History) London.

NICHOL, CHARLES A., Senior Instructor in Pharmacology, Western Reserve School of Medicine.
NIEUWKOOP, PIETER D., Research Fellow, Hubrecht Lab., Utrecht, Netherlands.
OSTERHOUT, W. J. V., Member, *Emeritus*, Rockefeller Institute.
PARMENTER, CHARLES L., Professor of Zoology, University of Pennsylvania.
PARPART, ARTHUR K., Chairman, Department of Biology, Princeton University.
PERKINS, JOHN F., Assistant Professor of Physiology, University of Chicago.
PERSON, PHILIP, USPHS Post Doctoral Research Fellow, Rutgers University.
PETTIBONE, MARIAN H., Research Associate, Johns Hopkins University.
PIERCE, MADELENE E., Professor of Zoology, Vassar College.
PROCTOR, NATHANIEL K., Associate Professor of Biology, Morgan State College.
PROSSER, C. LADD, Professor of Physiology, University of Illinois.
RATTENBURY, JOAN C., Lecturer in Zoology, McGill University.
RAY, DAVID T., Instructor of Zoology, Howard University.
REVERBERI, GIUSEPPE, Professor of Zoology, Universita Palermo, Italy, via Archirafi,18.
RISER, NATHAN W., Associate Professor of Biology, Fisk University.
RONKIN, R. R., Assistant Professor of Physiology, University of Delaware.
ROOFE, PAUL G., Chairman and Professor of Anatomy, University of Kansas.
ROSE, S. MERYL, Associate Professor of Zoology, University of Illinois.
ROTH, JAY S., Assistant Professor of Biochemistry, Hahnemann Medical College and Hospital.
RUGH, ROBERTS, Associate Professor of Radiology, Columbia University.
SANDEEN, MURIEL I., Instructor in Zoology, Duke University.
SCHAEFFER, ASA A., Professor of Biology, Temple University and Biological Institute.
SCHARRER, ERNST A., Associate Professor, University of Colorado School of Medicine.
SCHECHTER, VICTOR, Assistant Professor of Biology, The City College of New York.
SCLUFER, EVELYN, Graduate Student, University of Pennsylvania.
SCOTT, SISTER FLORENCE MARIE, Professor of Biology, Seton Hill College.
SCOTT, ALLAN, Professor of Biology, Colby College.
SCOTT, DWIGHT B. MCNAIR, Associate in Biochemistry, Children's Hospital of Philadelphia.
SHANES, A. M., Physiologist, National Institutes of Health.
SHAVER, JOHN, Assistant Professor of Zoology, University of Missouri.
SICHEL, F., Professor of Physiology and Biophysics, University of Vermont, College of Medicine.
SILVA, PAUL C., Research Associate in Botany, University of California.
SLIFER, ELEANOR H., Associate Professor of Zoology, State University of Iowa.
SOLOMON, SIDNEY, Instructor in Physiology, Medical College of Virginia.
SONNENBLICK, B. P., Associate Professor of Biology, Rutgers University.
SPEIDEL, CARL C., Professor and Chairman, School of Anatomy, University of Virginia.
SPRATT, NELSON T., JR., Professor of Zoology, University of Minnesota.
STADLER, DAVID R., Instructor in Biology, University of Rochester.
STOKEY, ALMA C., Professor of Plant Science, *Emeritus*, Mount Holyoke College.
STRITTMATTER, CORNELIUS F., Harvard Medical School.
STURTEVANT, A. H., Professor of Biology, California Institute of Technology.
TAYLOR, WILLIAM RANDOLPH, Professor of Botany, University of Michigan.
TOBIAS, JULIAN M., Associate Professor of Physiology, University of Chicago.
TRACY, HENRY C., Professor of Anatomy, *Emeritus*, University of Kansas.
TRAGER, WILLIAM, Associate Member, Rockefeller Institute.
TRINKAUS, J. P., Assistant Professor of Zoology, Yale University.
TRUANT, ALDO P., Assistant Professor of Pharmacology, Tufts Medical School.
WARNER, ROBERT C., Assistant Professor of Chemistry, New York University College of Medicine.
WATTERSON, RAY L., Associate Professor of Biology, Northwestern University.
WEBB, H. MARGUERITE, Assistant Professor of Biology, Boston College.
WHITING, P. W., Professor of Zoology, University of Pennsylvania.
WICHTERMAN, RALPH, Professor of Biology, Temple University.
WILBER, CHARLES G., Director, Biological Laboratories, St. Louis University.
WILSON, WALTER L., Instructor of Physiology and Biophysics, University of Vermont.
WRIGHT, PAUL A., Assistant Professor of Zoology, University of Michigan.

WRINCH, DOROTHY, Lecturer in Physics, Smith College.
ZWILLING, EDGAR, Associate Professor, University of Connecticut.
YAMADA, TUNEO, Nagoya University, Japan.

**Beginning Investigators, 1952**

ALLEN, ROBERT DAY, Graduate Student, University of Pennsylvania.
BEISER, WILLIAM, JR., Student, University of Delaware.
BERNSTEIN, GERALD S., Graduate Student, University of Delaware.
BUTROS, JOSEPH, Graduate Student, Emory University.
CHAET, ALFRED B., Research Assistant, University of Pennsylvania.
COLLIER, JACK R., Graduate Student, University of North Carolina.
CONOVER, JOHN TOWNSEND, Graduate Assistant, University of Michigan.
CONWAY, JOHN V., Student, University of Delaware.
COUILLARD, PIERRE, Graduate Student, University of Pennsylvania.
DECK, J. DAVID, Graduate Student, Princeton University.
GOLDSTEIN, LESTER, Graduate Student, University of Pennsylvania.
GRAVES, ROBERT C., Graduate Student, Northwestern University.
GREENE, PETER HAROLD, University Fellow in Mathematical Biology, University of Chicago.
GROSS, PAUL R., Assistant Instructor, University of Pennsylvania.
HEMPLING, HAROLD G., Graduate Student, Princeton University.
HERBERT, EDWARD, Graduate Student, University of Pennsylvania.
HERR, EARL B., JR., Graduate Student, University of Delaware.
HINTON, CLAUDE W., Graduate Student, California Institute of Technology.
HOFFMAN, JOSEPH F., Graduate Student, Princeton University.
HONEGGER, CAROL M., Instructor, Temple University.
HOROWITZ, IONA B., Student, Vassar College.
IVEY, WILLIAM D., Graduate Student, Emory University.
KATZ, ARNOLD M., University of Chicago.
KOHN, ROBERT R., Teaching Fellow, University of Michigan.
LANDAU, JOSEPH V., Research Assistant, New York University.
MCCANN, FRANCES, Graduate Student, University of Connecticut.
MCCAULEY, MOLLY M., Graduate Student, St. Louis University.
MARONEY, SAMUEL P., Graduate Student, University of Delaware.
MEYER, MARION P., Research Assistant, University of Wisconsin.
MONER, JOHN G., JR., Graduate Student, Princeton University.
PADYKULA, HELEN A., Research Fellow, Harvard Medical School.
PANDALAI, KRISHNAN R., Assistant Professor, Medical College, Trivandrum, S. India.
ROTHMAN, WALTER H., Research Assistant, University of Wisconsin.
SHANKLIN, DOUGLAS R., Student, New York State College of Medicine at Syracuse.
SLATER, JOHN V., Postdoctorate Fellow A.E.C., University of Michigan.
STEINBERG, MALCOLM S., Graduate Student, Amherst College.
WHITEACRE, JAMES F., Instructor in Physiology, Mount Holyoke College.
WOLF, BARBARA, Assistant, Rockefeller Institute.

**Library Readers, 1952**

ANDRUS, JULIAN L., Associate Professor of Pharmacology, Philadelphia College of Pharmacology.
BAUMBERGER, J. PERCY, Professor of Physiology, Stanford University.
BODANSKY, OSCAR, Attending Clinical Biochemist, Sloan-Kettering Institute.
COWGILL, ROBERT W., Instructor in Biochemistry, Washington University School of Medicine.
D'ANCONA, UMBERTO, Professor, Inst. of Zoology, University of Padua, Italy.
DALY, MARIE M., Assistant, Rockefeller Institute for Medical Research.
DEANE, HELEN W., Assistant Professor of Anatomy, Harvard University Medical School.
DIXON, FRANK J., Professor of Pathology, University of Pittsburgh, School of Medicine.
DORFMAN, ALBERT, Assistant Professor of Pediatrics, University of Chicago.

FREUND, JULES, Chief, Division of Applied Immunology, Public Health Research Institute.
FRIES, E. F. B., Assistant Professor, The City College of New York.
GABRIEL, MORDECAI L., Assistant Professor, Brooklyn College.
GAFFRON, HANS, Associate Professor of Biochemistry, University of Chicago.
GINSBERG, HAROLD S., Associate Professor, Western Reserve University School of Medicine.
GROBSTEIN, CLIFFORD, Biologist, National Cancer Institute.
GUREWICH, VLADIMIR, Assistant Visiting Physician, Bellevue Hospital.
JOHNSON, FRANK H., Associate Professor of Biology, Princeton University.
HODES, ROBERT, Professor of Neurophysiology, Tulane University School of Medicine.
JONES, SARAH R., Instructor in Zoology, Connecticut College.
KABAT, ELVIN A., Associate Professor of Bacteriology, Columbia University.
KARUSH, FRED, Assistant Professor of Immunology, Univ. of Pennsylvania School of Medicine
KIEBEL, GERALDINE, Public Health Service Predoctorate Research Fellow, New York University.
KRAMER, MOLLIE P., Bibliographer, American Meteorological Society.
KULKA, JOHANNES P., Instructor in Pathology, Harvard Medical School.
KURNICK, NATHANIEL B., Assistant Professor of Medicine, Tulane University School of Medicine.
LINDEGREN, CARL C., Professor, Southern Illinois University.
LOEWI, OTTO, Research Professor in Pharmacology, New York Univ. Bellevue Medical Center.
LOWENSTEIN, BERTRAND E., c/o Dade County Cancer Institute.
LURIA, S. E., Professor of Bacteriology, University of Illinois.
MCDONALD, SISTER ELIZABETH SETON, Chairman, Dept. of Biology, College of Mt. St. Joseph.
MACEY, ROBERT I., U. S. P. H. S. Research Fellow, University of Chicago.
NACHMANSOHN, DAVID, Associate Professor, Columbia University.
NAMIAS, JEROME, Chief, Extended Forecast Section, United States Weather Bureau.
REINER, JOHN M., Assistant Professor of Biochemistry, Columbia University.
ROYS, CHESTER, Research Associate, Tufts College.
SCHMIDT, GERHARD, Research Professor of Biochemistry, Tufts College Medical School.
SCOTT, THOMAS F. M., Research Professor of Pediatrics, Children's Hospital of Philadelphia.
SELBY, CECILY C., Assistant, Virus Study Section, Sloan-Kettering Institute.
SIPPEL, THEODORE O., Graduate Student, Yale University.
STOLOFF, LEONARD, Research Director, Seaplant Chemical Corporation.
STUNKARD, HORACE W., Professor of Biology, New York University.
SULKIN, S. EDWARD, Professor of Bacteriology, Southwestern Medical School.
TASAKI, ICHIJI, Research Associate, Central Institute for the Deaf, St. Louis.
TROTTER, MILDRED, Professor of Gross Anatomy, Washington University.
UHELES, R., Student, New York University.
WAINIO, WALTER W., Associate Professor of Biochemistry, Rutgers University.
WETMORE, RALPH, Department of Botany, Harvard University.
ZORZOLI, ANITA, Assistant Professor of Physiology, Southern Illinois University.

**Research Assistants, 1952**

ALTAMIRANO, MARIO, Columbia University.
BERGMAN, RONALD, University of Illinois.
BORYSKO, EMIL, Johns Hopkins University.
BULLOWA, ANNE, New York Medical College.
CLARK, MARGUERITE R., Western Reserve University.
COHEN, MAX, Columbia University.
COOK, JOHN S., Princeton University.
DRAKE, JOHN W., Yale University.
ENGELS, AUDREY, University of Minnesota.
EWALL, LAROY H., Fisk University.
FASS, JEROME, Rockefeller Institute.
FINGERMAN, MILTON, Northwestern University.
FOX, HOWARD A., Union College.

FRANKLIN, MALCOLM, University of Mississippi.
FRICK, WILLY, Institute for Muscle Research.
GALL, JOSEPH G., Yale University.
GLADE, RICHARD W., University of Illinois.
GROESBECK, MARJORIE, University of Pennsylvania.
HAYWARD, HUGH R., Oberlin College.
HORVATH, BENI, Carnegie Institution.
HOWARD, ROBERT S., Northwestern University.
JACOBS, JAY A. H., Johns Hopkins University.
JOLY, AYLTHON B., University of Michigan.
KAO, CHUN-JUAN, Vassar College.
KAPLAN, ANN E., Columbia University.
KAYE, ALVIN M., Columbia University.
LOOS, GORDON M., Harvard University.
MARTIN, B. JEAN, University of Minnesota.
MELTON, C. E., University of Illinois.
ROSENBLÜTH, MICHAEL, New York University Medical School.
ROSENBLUTH, RAJA, Columbia University.
ROTH, LORRAINE B., Children's Hospital of Philadelphia.
SABAL, JAMES, University of Michigan.
SHIRER, HAMPTON W., Naval Medical Research Institute.
STAUB, NORMAN C., State University School of Medicine at Syracuse.
STEARNS, RICHARD N., University of Illinois.
SULLIVAN, ROBERT L., North Carolina State College.
VAUGHAN-WILLIAMS, E. M., Wilmer Inst., Johns Hopkins Hospital.
YARUS, STANLEY S., Hahnemann Medical College.
YOUNGS, LILLIAN M., University of North Carolina.

**Students, 1952**

BOTANY

ALLEN, MARJORY A., Smith College.
ANDERSON, SONYA, University of Massachusetts.
ARNDT, WILLIAM F., JR., Wesleyan University.
ATKIN, ADAM, Antioch College.
CORMIER, JOAN, Elmira College.
DUBÉ, JEAN F., Yale University.
HAWTHORNE, MARY E., Rhode Island University.
KROTKOV, N. SONIA, Queen's University.
MARKO, ANITA R., Adelphi College.
ROMIG, ROBERT W., Drew University.
STEIN, JANET R., Wellesley College.

ECOLOGY

BERNATOWICZ, ALBERT J., National Science Foundation.
FAHEY, ELIZABETH M., State Teachers College.
GOULD, DAVID, Bard College.
HALL, JOHN E., University of New Hampshire.
HOWSON, MARY CHRISTINE, Vassar College.
POLNIK, AMELIA, University of Chicago.
SCHELTEMA, RUDOLF S., George Washington University.

EMBRYOLOGY

ABRAMS, LEONARD S., Columbia University.
AMBERSON, BARBARA D., Jefferson Medical College.
BECKER, SIMON C., St. Norbert College.

CHILD, FRANK M., III, Amherst College.
CHIN, EDWARD, University of New Hampshire.
COHEN, MOLLIE, Brooklyn College.
DOAK, ANNE B., Randolph-Macon Woman's College.
FIMIAN, WALTER J., JR., University of Notre Dame.
GAUTEREAUX, SISTER MIRIAM EUGENE, Marquette University.
GOLDMAN, ALLEN SEYMOUR, Brown University.
HEY, ROBERT VANDE, St. Norbert College.
HILL, ROBERT B., Tufts College.
HILLMAN, RALPH, Yale University.
ITO, SUSUMU, Western Reserve University.
KING, ELIZABETH N., Wellesley College.
KRIVANEK, JEROME O., University of Florida.
LOWRY, JOAN L., Wellesley College.
MCKENZIE, JOHN W., University of North Carolina.
MORRIS, CHARLES C., Columbia University.
RAFFERTY, KEEN A., JR., University of Illinois.
SAXER, ROBERT J., Spring Hill College.
VANGELDER, RICHARD G., University of Illinois.
WARREN, MARTHA M., Mount Holyoke College.

## PHYSIOLOGY

BACILA, METRY, Instituto de Biologia, Rua Angelo Sampaio, Brasil.
BAKER, HINTON J., United States Army.
BENNETT, MICHAEL V., Yale University.
BENNETT, THEDA E., Syracuse University.
BOGORAD, LAWRENCE, Rockefeller Institute for Medical Research.
BORYSKO, EMIL, Johns Hopkins University.
BRADFORD, SARAH C., McGuire Veterans Administration Hospital.
BURKE, WILLIAM T., University of Rochester.
CASTOR, LAROY N., University of Pennsylvania.
CEAS, MARIA PIERA, Columbia University.
CHALFIN, DAVID, Florida State University.
COVELL, GERALDINE M., University of Rochester.
DAVIDHEISER, ROGER, Indiana University.
DEGRAFF, ARTHUR C., New York University College of Medicine.
EPSTEIN, RICHARD H., University of Rochester.
FENN, PRISCILLA, University of Rochester.
HURLBERT, ROBERT B., University of Wisconsin.
MACLEAN, ELIZABETH C., Mt. Holyoke College.
MCNAMARA, DORRIS M., Rockefeller Institute for Medical Research.
MENZ, LEO J., St. Louis University.
OVERTON, JANE H., University of Chicago.
PECK, HARRY D., JR., Wesleyan University.
PUNNETT, THOMAS R., University of Illinois.
ROSLANSKY, JOHN D., University of Washington.
SMITH, CHARLES E., University of Illinois.
STOIBER, ALMA MARY, University of Michigan.
WEIS, DALE, Yale University.

## INVERTEBRATE ZOOLOGY

BATTIN, WILLIAM, University of Minnesota.
BEQUAERT, HELEN M., Cornell University.
BERKOWITZ, HAROLD, Amherst College.
BLASZCZYNSKI, HENRY J., St. Louis University.
BLOEDEL, PRENTICE, University of California.

CASTLEMAN, MARY, Randolph-Macon Woman's College.
CONTE, ANTHONY R., Fordham University.
DARLINGTON, JULIAN T., University of Florida.
EINHORN, IRVING N., Temple University.
FLAVIN, JOHN W., Brown University.
FOSTER, JOYCE M., DePauw University.
FOSTER, KENDALL W., JR., Tufts College.
FRIEDMAN, FRANK, New York University.
GALE, MARTHA G., University of Wisconsin.
GALLICCHIO, VINCENT, University of Illinois.
GAUMER, ALBERT E., Purdue University.
GILLIGAN, JOSEPH I., Villanova College.
GLADE, RICHARD W., University of Illinois.
GLASSMAN, EDWARD, Johns Hopkins University.
GOJMERAC, WALTER L., Marquette University.
GRABOWSKI, CASIMER T., Johns Hopkins University.
HAMMEN, CARL S., St. John's College.
HANZELY, JOSEPH B., Catholic University of America.
HERZENBERG, LEONARD A., Brooklyn College.
HISAW, FREDERICK L., Harvard University.
HUNTINGTON, NANCY, Drew University.
KAMEMOTO, FRED I., Purdue University.
KOHN, VILMA LAVETTI, University of Michigan.
KOSTYO, JACK L., Oberlin College.
LEAVENWORTH, MARTHA, Carleton College.
LUYTEN, MONA, Oberlin College.
LYTLE, CHARLES F., Wabash College.
MCMAHON, JEAN, Fordham University.
MCMAHON, RITA MARY, Fordham University.
MAGUIRE, BASSETT, Cornell University.
MAGUIRE, MARJORIE P., Cornell University.
MARQUARDT, WILLIAM C., University of Illinois.
MILKMAN, ROGER D., Harvard University.
MYERS, ROSE M., Purdue University.
NICELY, MARTHA, Radcliffe College.
PEGAU, LUCY B., Oberlin College.
PRIMACK, NATHAN, Purdue University.
RAWSON, KENNETH S., Cornell University.
ROBERTSON, SALLY JEAN, Randolph-Macon Woman's College.
ROSS, FRANCES, Vassar College.
RUDNYANSKY, BERTINA M., Indiana University.
SCHIFF, JEROME A., Brooklyn College.
SCIARRA, JOHN J., Yale University.
SILVERMAN, MARIAN J., Drew University.
SMALL, MARCIA C., University of Massachusetts.
TANNER, WILMA, Yale University.
THOMAS, HILDA P., Austin College.
WALKER, JANE A., Smith College.
WESTFALL, JANE A., Lafayette, California.
WOLK, ROBERT G., The City College of New York.

### 4. LALOR FELLOWS, 1952

BARTON, JAY, II, Columbia University.
BOYER, PAUL D., University of Minnesota.
CIERESZKO, LEON, University of Oklahoma.
CONNELLY, CLARENCE M., Johns Hopkins University.

FLORKIN, MARCEL, University of Liège, Belgium.
FREEMAN, PAUL J., San Jose State College.
INOUÉ, SHINYA, University of Washington School of Medicine.
NICHOL, CHARLES A., Western Reserve University School of Medicine.
STRITTMATTER, CORNELIUS F., Harvard University Medical School.

## 5. TABULAR VIEW OF ATTENDANCE, 1948–1952

| | *1948* | *1949* | *1950* | *1951* | *1952* |
|---|---|---|---|---|---|
| INVESTIGATORS—Total | 326 | 344 | 338 | 303 | 300 |
| Independent | 183 | 193 | 198 | 186 | 172 |
| Under Instruction | 42 | 52 | 43 | 28 | 38 |
| Library Readers | 50 | 55 | 48 | 37 | 49 |
| Research Assistants | 51 | 44 | 49 | 52 | 47 |
| STUDENTS—Total | 123 | 128 | 126 | 124 | 123 |
| Zoology | 54 | 55 | 55 | 55 | 55 |
| Embryology | 29 | 31 | 29 | 27 | 23 |
| Physiology | 25 | 27 | 27 | 29 | 27 |
| Botany | 15 | 15 | 13 | 15 | 11 |
| Ecology | | | | | 7 |
| TOTAL ATTENDANCE | 449 | 472 | 444 | 427 | 423 |
| Less persons registered as both students and investigators | 6 | 2 | | | 2 |
| | 443 | 470 | 444 | 427 | 422 |
| INSTITUTIONS REPRESENTED—Total | 158 | 155 | 156 | 158 | 149 |
| By Investigators | 117 | 114 | 114 | 115 | 92 |
| By Students | 68 | 68 | 67 | 43 | 57 |
| SCHOOLS AND ACADEMIES REPRESENTED | | | | | |
| By Investigators | 1 | 1 | 2 | 1 | 1 |
| By Students | | | | | 3 |
| FOREIGN INSTITUTIONS REPRESENTED | | | | | |
| By Investigators | 8 | 6 | 6 | 8 | 7 |
| By Students | 4 | 3 | 2 | 3 | 2 |

## 6. COOPERATING AND SUBSCRIBING INSTITUTIONS, 1952

### Cooperating Institutions

Amherst College
Brooklyn College
Bryn Mawr College
Children's Hospital of Philadelphia
College of Mt. St. Joseph-on-the-Ohio
College of Physicians and Surgeons
Columbia University
Drew University
Duke University
Elmira College
Fordham University
Harvard University
Harvard University Medical School
Institute for Muscle Research
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly and Company
Morgan State College
National Cancer Institute
New York University College of Medicine
New York University, Heights
New York University, Washington Square College
North Carolina State College of Agriculture and Engineering
Northwestern University
Oberlin College
Princeton University
Rockefeller Institute for Medical Research
Rutgers University
Saint Louis University
Smith College
Southwestern Medical College
State University of Iowa
State University of New York College of Medicine
Temple University
Tufts College Medical School

Tulane University School of Medicine
University of Chicago
University of Connecticut
University of Delaware
University of Illinois
University of Kansas
University of Maryland School of Medicine
University of Massachusetts
University of Michigan
University of Minnesota
University of New Hampshire
University of Pennsylvania
University of Pennsylvania Medical School
University of Rochester
University of Vermont Medical School
University of Virginia
University of Wisconsin
Vassar College
Washington University School of Medicine
Wesleyan University
Western Reserve University School of Medicine
Yale University

**Subscribing Institutions**

Boston Dispensary
Brown University
California Institute of Technology
Carnegie Embryological Laboratory
Central Institute for the Deaf
City College of New York
Colby College
Cornell University
Cornell University Medical College
Emory University
George Washington University
Hahnemann Medical School
Marquette University
Massachusetts General Hospital
Mount Holyoke College
National Medical Research Institute
National Science Foundation
New York Medical College
Oak Ridge Institute for Nuclear Studies, Inc.
Public Health Research Inst. of New York City
Rockefeller Foundation
St. John's College
St. Norbert College
Seaplant Chemical Corporation
Sloan-Kettering Institute
Spring Hill College
Tufts College
University of California
University of Colorado School of Medicine
University of Florida
University of Mississippi
University of North Carolina
University of Pittsburgh
University of South Dakota
Wellesley College

## 7. Evening Lecturers, 1952

June 27
I. Tasaki ..... "A survey of the development of our concept of nervous conduction."

July 4
H. F. Blum ..... "Ultraviolet light and cancer."

July 11
K. V. Thimann ..... "The physiology of cell elongation in plant tissues."

July 18
Otto Loewi ..... "From the workshop of discoveries."

July 25
Marcel Florkin ..... "Some biochemical implications of adaptation in animals."

August 1
J. S. Nicholas ..... "The action of cells and aggregates in embryonic formation."

August 8
Thurlow Nelson ..... "Some studies of the ecology and physiology of the oyster."

August 15
JOHN H. WELSH ..... "The acetylcholine system."
August 22
D. W. WOOLLEY ..... "Antimetabolites in relation to natural processes and man's control of them."

## 8. TUESDAY EVENING SEMINARS, 1952

Tuesday, July 1
PAUL R. GROSS ..... "An enzymatic study of yolk-platelet lysis."
ALFRED B. CHAET ..... "Action of heparin preparations on cells and tissues."
L. V. HEILBRUNN, ATIDA HALABAN, WALTER L. WILSON ..... "An attempt to influence the survival time of mice with implanted tumors."

Tuesday, July 8
S. M. ROSE ..... "The specific suppression of differentiation of embryonic heart, blood, and brain by the like adult tissues."
JOHN R. SHAVER ..... "Some studies in the initiation of cleavage in the frog egg."
CHARLES B. METZ, DOROTHY R. PITELKA ..... "The structure of the neuromotor apparatus of Paramecium as revealed by the electron microscope."

Tuesday, July 15
ROBERT D. ALLEN ..... "A visible response to stimulation in egg cells of the surf clam."
DANIEL MAZIA, KATSUMA DAN ..... "Isolation and biochemical characterization of the mitotic apparatus of dividing cells."
J. T. BONNER ..... "Mitotic activity in relation to differentiation in the slime mold, *Dictyostelium discoideum*."
ETHEL BROWNE HARVEY ..... "Electrical method of sexing Arbacia and obtaining small quantities of eggs repeatedly."

Tuesday, July 22
BERTA SCHARRER ..... "Further studies of the intercerebralis-cardiacum-allatum system of insects."
ERNST SCHARRER ..... "The neurohypophysis of elasmobranch fishes."
CARL C. SPEIDEL ..... "Balantidial infection in tadpoles as recorded by cine-photomicrography."

Tuesday, July 29
GERALD S. BERNSTEIN ..... "Sperm agglutinins in the egg jelly of the frogs, *Rana pipiens* and *R. clamitans*."
G. S. FRAENKEL ..... "A function of the salivary glands of the larvae of Drosophila and other flies."
FRANCIS HAXO ..... "Carotenoid formation in mutant strains of *Neurospora crassa*."

Tuesday, August 5
ANDREW G. SZENT-GYORGYI ..... "Meromyorsin, the sub-units of myoris."

WILLIAM H. JOHNSON ..... "Studies on the viscoelastic properties of smooth muscle."
C. LADD PROSSER ..... "Conduction in non-striated muscles."

Tuesday, August 12
PHILIP PERSON, W. W. WAINIO, B. EICHEL ..... "The partial separation of the prosthetic groups of cytochrome *b* and cytochrome oxidase from active enzyme."
DWIGHT B. MCNAIR SCOTT ..... "The oxidative pathway of carbohydrate metabolism in *E. coli*."
JAY S. ROTH ..... "Ribonucleases."

Tuesday, August 19
PHILIP B. ARMSTRONG ..... "Function in the developing liver."
SEYMOUR H. WOLLMAN, EDGAR ZWILLING ..... "Radioiodine uptake in the chick embryo thyroid gland."
RAY L. WATTERSON ..... "The glycogen body as a derivative of the avian neural tube."

## 9. MEMBERS OF THE CORPORATION, 1952

### 1. LIFE MEMBERS

BECKWITH, DR. CORA J., Vassar College, Poughkeepsie, New York.
BILLINGS, MR. R. C., 66 Franklin Street, Boston, Massachusetts.
CALVERT, DR. PHILIP P., University of Pennsylvania, Philadelphia, Pennsylvania.
COLE, DR. LEON J., College of Agriculture, Madison, Wisconsin.
CONKLIN, PROF. EDWIN G., Princeton University, Princeton, New Jersey.
COWDRY, DR. E. V., Washington University, St. Louis, Missouri.
DUNGAY, DR. NEIL S., Carleton College, Northfield, Minnesota.
JACKSON, MR. CHAS. C., 24 Congress Street, Boston, Massachusetts.
JACKSON, MISS M. C., 88 Marlboro Street, Boston, Massachusetts.
KING, MR. CHAS. A.
KING, DR. HELEN D., Wistar Inst. of Anatomy and Biology, Philadelphia, Pennsylvania.
LEWIS, PROF. W. H., Johns Hopkins University, Baltimore, Maryland.
LOWTHER, DR. FLORENCE DEL., Barnard College, New York City, New York.
MACNAUGHT, MR. FRANK M., Woods Hole, Massachusetts.
MEANS, DR. J. H., 15 Chestnut Street, Boston, Massachusetts.
MOORE, DR. GEORGE T., Missouri Botanical Gardens, St. Louis, Missouri.
MOORE, DR. J. PERCY, University of Pennsylvania, Philadelphia, Pa.
NOYES, MISS EVA J.
PAYNE, DR. FERNANDUS, Indiana University, Bloomington, Indiana.
PORTER, DR. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
SCOTT, DR. ERNEST L., Columbia University, New York City, New York.
SEARS, DR. HENRY F., 86 Beacon Street, Boston, Massachusetts.
SHEDD, MR. E. A.
WAITE, PROF. F. C., 144 Locust Street, Dover, New Hampshire.
WALLACE, LOUISE B., 359 Lytton Avenue, Palo Alto, California.

2. Regular Members

Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts.
Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
Adolph, Dr. Edward F., University of Rochester School of Medicine and Dentistry, Rochester, N. Y.
Albaum, Dr. Harry G., Biology Department, Brooklyn College, Brooklyn, New York.
Albert, Dr. Alexander, Mayo Clinic, Rochester, Minnesota.
Allee, Dr. W. C., 114 Leigh Hall, University of Florida, Gainesville, Florida.
Alscher, Dr. Ruth, Dept. of Physiology, Manhattanville College of the Sacred Heart, Purchase, New York.
Amberson, Dr. William R., Dept. of Physiology, University of Maryland School of Medicine, Baltimore, Md.
Anderson, Dr. Rubert S., Dept. of Physiology, University of South Dakota, Vermillion, S. D.
Anderson, Dr. T. F., University of Pennsylvania, Philadelphia, Pennsylvania.
Angerer, Dr. C. A., Department of Physiology, Ohio State University, Columbus, Ohio.
Armstrong, Dr. Philip B., State University of New York, College of Medicine, Syracuse 10, New York.
Atwood, Dr. Kimball C., 68½ Outer Drive, Oak Ridge, Tennessee.
Austin, Dr. Mary L., Wellesley College, Wellesley, Massachusetts.
Ayers, Dr. John C., Department of Oceanography, Cornell University, Ithaca, New York.
Baitsell, Dr. George A., 54 Hill House Ave., Connecticut.
Baker, Dr. H. B., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.
Ball, Dr. Eric G., Dept. of Biological Chemistry, Harvard University Medical School, Boston 15, Massachusetts.
Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire.
Bard, Dr. Philip, Johns Hopkins Medical School, Baltimore, Maryland.
Barron, Dr. E. S. G., Department of Medicine, University of Chicago, Chicago, Illinois.
Barth, Dr. L. G., Department of Zoology, Columbia University, New York City, New York.
Bartlett, Dr. James H., Department of Physics, University of Illinois, Urbana, Illinois.
Beams, Dr. Harold W., Department of Zoology, State University of Iowa, Iowa City, Iowa.
Beck, Dr. L. V., Dept. of Physiology and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pennsylvania.
Beers, Dr. C. D., University of North Carolina, Chapel Hill, North Carolina.
Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, Louisiana.
Bertholf, Dr. Floyd M., College of the Pacific, Stockton, California.
Bevelander, Dr. Gerrit, New York University School of Medicine, New York City, New York.

BIGELOW, DR. HENRY B., Museum of Comparative Zoology, Harvard University, Cambridge, Mass.

BIGELOW, DR. ROBERT P., Massachusetts Institute of Technology, Cambridge, Massachusetts.

BISHOP, DR. DAVID W., Dept. of Embryology, Carnegie Inst. of Washington, Baltimore 5, Maryland.

BLANCHARD, DR. K. C., Johns Hopkins Medical School, Baltimore, Maryland.

BLUM, DR. HAROLD F., Department of Biology, Princeton University, Princeton, New Jersey.

BODANSKY, DR. OSCAR, Dept. of Biochemistry, Sloan-Kettering Division, Cornell University Medical College, New York City, New York.

BODINE, DR. J. H., Department of Zoology, State University of Iowa, Iowa City, Iowa.

BODIAN, DR. DAVID, Department of Epidemiology, Johns Hopkins University, Baltimore 5, Maryland.

BOELL, DR. EDGAR J., Yale University, New Haven, Connecticut.

BOETTIGER, DR. EDWARD G., Department of Zoology, University of Connecticut, Storrs, Conn.

BONNER, DR. JOHN T., Department of Biology, Princeton University, Princeton, New Jersey.

BRADLEY, DR. HAROLD C., 2639 Durant Avenue, Berkeley 4, California.

BRODIE, MR. DONALD M., 522 Fifth Avenue, New York 18, New York.

BRONFENBRENNER, DR. J. J., 1703 Reynolds Road, Winston-Salem, North Carolina.

BRONK, DR. DETLEV W., Johns Hopkins University, Baltimore, Maryland.

BROOKS, DR. MATILDA M., University of California, Dept. of Physiology, Berkeley 4, California.

BROWN, DR. FRANK A., JR., Department of Biological Sciences, Northwestern University, Evanston, Illinois.

BROWN, DR. DUGALD E. S., Department of Zoology, University of Michigan, Ann Arbor, Michigan.

BROWNELL, DR. KATHERINE A., Ohio State University, Columbus, Ohio.

BUCK, DR. JOHN B., Laboratory of Physical Biology, National Institutes of Health, Bethesda, Md.

BUCKINGHAM, MISS EDITH N., Sudbury, Massachusetts.

BUDINGTON, DR. R. A., Box 954, Winter Park, Florida.

BULLINGTON, DR. W. E., Randolph-Macon College, Ashland, Virginia.

BULLOCK, DR. T. H., University of California, Los Angeles 24, California.

BURBANCK, DR. WILLIAM D., Box 834, Emory University, Georgia.

BURDICK, DR. C. LALOR, The Lalor Foundation, Lancaster Pike and Old Baltimore Road, Wilmington, Delaware.

BURKENROAD, DR. M. D., Institute of Marine Science, Port Arkansas, Texas.

BUTLER, DR. E. G., Princeton University, Princeton, New Jersey.

CAMERON, DR. J. A., Baylor College of Dentistry, Dallas, Texas.

CANNAN, DR. R. K., National Research Council, 2101 Constitution Avenue, Washington N.W., D. C.

CANTONI, DR. GIULIO, Department of Pharmacology, Western Reserve University, Cleveland 9, Ohio.

CARLSON, DR. A. J., Department of Physiology, The University of Chicago, Chicago 37, Illinois.
CAROTHERS, DR. E. ELEANOR, 134 Avenue C., East Kingman, Kansas.
CARPENTER, DR. RUSSELL L., Tufts College, Medford 55, Massachusetts.
CARSON, MISS RACHEL, 204 Williamsburg Drive, Silver Spring, Maryland.
CARVER, DR. GAIL L., Mercer University, Macon, Georgia.
CATTELL, DR. MCKEEN, Cornell University Medical College, 1300 York Avenue, New York City, N. Y.
CATTELL, MR. WARE, Cosmos Club, Washington 5, D. C.
CHAMBERS, DR. EDWARD, Medical Research Foundation of Dade County, 1800 N.W. 10th Avenue, Miami 36, Florida.
CHAMBERS, DR. ROBERT, Chief Research Consultant, Dade County Cancer Inst., 1155 N.W. 15th Street, Miami, Florida.
CHARLES, DR. DONALD R., Dept. of Zoology, Division of Biological Sciences, University of Rochester, Rochester 3, New York.
CHASE, DR. AURIN M., Department of Biology, Princeton University, Princeton, New Jersey.
CHENEY, DR. RALPH H., Biology Department, Brooklyn College, Brooklyn 10, New York.
CHILD, DR. C. M., Jordan Hall, Stanford University, Stanford, California.
CHURNEY, DR. LEON, Dept. of Physiology, Louisiana State University School of Medicine, New Orleans, La.
CLAFF, MR. C. LLOYD, 5 Van Beal Road, Randolph, Massachusetts.
CLARK, DR. A. M., Department of Biology, University of Delaware, Newark, Delaware.
CLARK, DR. E. R., The Wistar Inst., Woodland Avenue and 36th Street, Philadelphia 4, Penn.
CLARK, DR. LEONARD B., Department of Biology, Union College, Schenectady, New York.
CLARKE, DR. GEORGE L., Harvard University, Biological Laboratory, Cambridge 38, Massachusetts.
CLELAND, DR. RALPH E., Indiana University, Bloomington, Indiana.
CLEMENT, DR. A. C., Department of Biology, Emory University, Emory, Georgia.
CLOWES, DR. G. H. A., Eli Lilly and Company, Indianapolis, Indiana.
COE, DR. W. R., Scripps Institute of Oceanography, La Jolla, California.
COHEN, DR. SEYMOUR S., Dept. of Physiological Chemistry, Univ. of Pennsylvania, Philadelphia, Penn.
COHN, DR. EDWIN J., 183 Brattle Street, Cambridge 38, Massachusetts.
COLE, DR. ELBERT C., Department of Biology, Williams College, Williamstown, Massachusetts.
COLE, DR. KENNETH S., Naval Medical Research Institute, Bethesda 14, Maryland.
COLLETT, DR. MARY E., Western Reserve University, Mather College, Cleveland, Ohio.
COLTON, DR. H. S., Box 601, Flagstaff, Arizona.
COLWIN, DR. ARTHUR L., Department of Biology, Queens College, Flushing, New York.

COLWIN, DR. LAURA H., Department of Biology, Queens College, Flushing, New York.

COOPER, DR. KENNETH W., Department of Biology, Princeton University, Princeton, New Jersey.

COOPERSTEIN, DR. SHERWIN J., Department of Anatomy, Western Reserve University Medical School, Cleveland, Ohio.

COPELAND, DR. D. E., 1027 N. Manchester Street, Arlington 5, Virginia.

COPELAND, DR. MANTON, Bowdoin College, Brunswick, Maine.

COPLEY, DR. ALFRED L., Chargé de Recherches, Laboratoires de Recherches, Central International de L'Enfance, Chateande Longchamp, Paris, France.

CORNMAN, DR. IVOR, George Washington University, Warwick Memorial Clinic, 1339 H Street, N. W., Washington 5, D. C.

COSTELLO, DR. DONALD P., Dept. of Zoology, University of North Carolina, Chapel Hill, N. C.

COSTELLO, DR. HELEN MILLER, Dept. of Zoology, University of North Carolina, Chapel Hill, N. C.

CRAMPTON, DR. H. E., American Museum of Natural History, New York City, New York.

CRANE, MR. JOHN O., Woods Hole, Massachusetts.

CRANE, MRS. W. MURRAY, Woods Hole, Massachusetts.

CROASDALE, DR. HANNAH T., Dartmouth College, Hanover, New Hampshire.

CROUSE, DR. HELEN V., Goucher College, Baltimore, Maryland.

CROWELL, DR. P. S., JR., Department of Zoology, Indiana University, Bloomington, Indiana.

CURTIS, DR. MAYNIE R., University of Miami, Box 1015, South Miami, Florida.

CURTIS, DR. W. C., University of Missouri, Columbia, Missouri.

DAN, DR. KATSUMA, Misaki Biological Station, Misaki, Japan.

DANIELLI, DR. JAMES F., Department of Zoology, King's College, London, England.

DAWSON, DR. A. B., Harvard University, Cambridge, Massachusetts.

DAWSON, DR. J. A., The College of the City of New York, New York City, New York.

DEDERER, DR. PAULINE H., Connecticut College, New London, Connecticut.

DEMEREC, DR. M., Carnegie Inst. of Washington, Cold Spring Harbor, Long Island, New York.

DILLER, DR. IRENE C., Institute for Cancer Research, Philadelphia, Pennsylvania.

DILLER, DR. WILLIAM F., 2417 Fairhill Avenue, Glenside, Pennsylvania.

DODDS, DR. G. S., School of Medicine, West Virginia University, Morgantown, West Virginia.

DOLLEY, DR. WILLIAM L., University of Buffalo, Buffalo 14, New York.

DONALDSON, DR. JOHN C., University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

DOTY, DR. MAXWELL S., Department of Biology, University of Hawaii, Honolulu, T. H.

DRINKER, DR. CECIL K., Box 502, Falmouth, Massachusetts.

DUBOIS, DR. EUGENE F., Cornell Univ. Medical College, 1300 York Avenue, New York City, N. Y.

DUGGAR, DR. BENJAMIN M., Lederle Laboratories, Inc., Pearl River, New York.

DURYEE, DR. WILLIAM R., Carnegie Inst., 5241 Broad Branch Road, Washington 15, D. C.

EDDS, DR. MAC V., JR., Department of Biology, Brown University, Providence, Rhode Island.

EICHEL, DR. BERTRAM, Bureau of Biological Research, Box 515, Rutgers University, New Brunswick, N. J.

ELLIOTT, DR. ALFRED M., Department of Zoology, University of Michigan, Ann Arbor, Michigan.

EVANS, DR. TITUS C., State University of Iowa, Iowa City, Iowa.

FAILLA, DR. G., College of Physicians and Surgeons, New York City, New York.

FAURÉ-FREMIET, DR. EMMANUEL, Collège de France, Paris, France.

FERGUSON, DR. F. P., Department of Physiology, University of Maryland Medical School, Baltimore 1, Md.

FERGUSON, DR. JAMES K. W., Department of Pharmacology, University of Toronto, Ontario, Canada.

FIGGE, DR. F. H. J., University of Maryland, Medical School, Lombard and Green Streets, Baltimore 1, Maryland.

FINGERMAN, DR. MILTON, Department of Biological Sciences, Northwestern University, Evanston, Ill.

FISCHER, DR. ERNST, Dept. of Physiology, Medical College of Virginia, Richmond 19, Virginia.

FISHER, DR. JEANNE M., Department of Biochemistry, University of Toronto, Toronto, Canada.

FISHER, DR. KENNETH C., Department of Biology, University of Toronto, Toronto, Canada.

FORBES, DR. ALEXANDER, Biological Laboratories, Harvard University, Divinity Avenue, Cambridge, Massachusetts.

FRIES, DR. ERIK F. B., Department of Biology, City College of New York, New York City, N. Y.

FRISCH, DR. JOHN A., Canisius College, Buffalo, New York.

FURTH, DR. JACOB, 201 Delaware Avenue, Oak Ridge, Tennessee.

GABRIEL, DR. MORDECAI, Department of Biology, Brooklyn College, Brooklyn, New York.

GAFFRON, DR. HANS, Department of Biochemistry, University of Chicago, Chicago 37, Illinois.

GALTSOFF, DR. PAUL S., Woods Hole, Massachusetts.

GASSER, DR. HERBERT S., Director, Rockefeller Institute, New York City, New York.

GATES, DR. REGINALD R., Museum of Comparative Zoology, Oxford Street, Cambridge 38, Mass.

GEISER, DR. S. W., Southern Methodist University, Dallas, Texas.

GERARD, DR. R. W., Illinois Neuropsychiatric Inst., Chicago 12, Illinois.

GILMAN, DR. LAUREN C., Department of Zoology, University of Miami, Coral Gables, Florida.

GOLDFORB, DR. A. J., College of the City of New York, New York City, New York.

GOODCHILD, DR. CHAUNCEY G., Department of Biology, Emory University, Emory University, Georgia.

GOODRICH, DR. H. B., Wesleyan University, Middletown, Connecticut.
GOTTSCHALL, DR. GERTRUDE Y., 315 E. 68th Street, New York 21, New York.
GOULD, DR. H. N., Medical Sciences Information Exchange, 1113 Dupont Circle Bldg., Washington, D. C.
GRAND, MR. C. G., Dade County Cancer Inst., 1155 N.W. 15 St., Miami, Florida.
GRANT, DR. M. P., Sarah Lawrence College, Bronxville, New York.
GRAY, DR. IRVING E., Duke University, Durham, North Carolina.
GREEN, DR. JAMES W., Department of Physiology, Rutgers University, New Brunswick, New Jersey.
GREGORY, DR. LOUISE H., 1160 Fifth Avenue, New York City, New York.
GREGG, DR. J. R., Department of Zoology, Columbia University, New York 27, New York.
GROSCH, DR. DANIEL S., Division of Biological Sciences, Genetics, North Carolina State College, Raleigh, N. C.
GRUNDFEST, DR. HARRY, College of Physicians & Surgeons, Columbia University, New York City.
GUDERNATSCH, DR. FREDERICK, 41 Fifth Avenue, New York 3, New York.
GUTHRIE, DR. MARY J., Detroit Inst. for Cancer Research, 4811 John R Street, Detroit 1, Mich.
GUTTMAN, DR. RITA, Department of Physiology, Brooklyn College, Brooklyn, New York.
GUYER, DR. MICHAEL F., University of Wisconsin, Madison, Wisconsin.
HAGUE, DR. FLORENCE, Sweet Briar College, Sweet Briar, Virginia.
HAJDU, DR. STEPHEN, Research Associate, Institute for Muscle Research, Woods Hole, Mass.
HALL, DR. FRANK G., Duke University, Durham, North Carolina.
HAMBURGER, DR. VIKTOR, Department of Zoology, Washington University, St. Louis, Missouri.
HAMILTON, DR. HOWARD L., Iowa State College, Ames, Iowa.
HANCE, DR. ROBERT T., Box 108, R. R. No. 3, Loveland, Ohio.
HARMAN, DR. MARY T., Kansas State Agricultural College, Manhattan, Kansas.
HARNLY, DR. MORRIS H., Washington Square College, New York University, New York City, N. Y.
HARRISON, DR. ROSS G., Yale University, New Haven, Connecticut.
HARTLINE, DR. H. KEFFER, 215 Mergenthaler Hall, Johns Hopkins University, Baltimore 18, Md.
HARTMAN, DR. FRANK A., Hamilton Hall, Ohio State University, Columbus, Ohio.
HARVEY, DR. ETHEL BROWNE, 48 Cleveland Lane, Princeton, New Jersey.
HARVEY, DR. E. NEWTON, Guyot Hall, Princeton University, Princeton, New Jersey.
HAUSCHKA, DR. T. S., Inst. for Cancer Research, Experimental Zoology, Fox Chase, Philadelphia 11, Pennsylvania.
HAXO, DR. FRANCIS T., Division of Marine Botany, Scripps Institution of Oceanography, La Jolla, California.
HAYASHI, DR. TERU, Columbia University, New York City, New York.
HAYDEN, DR. MARGARET A., 34 Weston Road, Wellesley 81, Massachusetts.
HAYES, DR. FREDERICK R., Zoology Laboratory, Dalhousie University, Halifax, Nova Scotia.

HAYWOOD, DR. CHARLOTTE, Mount Holyoke College, South Hadley, Massachusetts.
HEILBRUNN, DR. L. V., Dept. of Zoology, University of Pennsylvania, Philadelphia, Pennsylvania.
HENDLEY, DR. CHARLES D., Dept. of Experimental Neurology, Tulane University, New Orleans, La.
HENLEY, DR. CATHERINE, Dept. of Zoology, University of North Carolina, Chapel Hill, N. C.
HENSHAW, DR. PAUL S., 17th Floor, 501 Madison Avenue, New York 22, New York.
HESS, DR. WALTER N., Hamilton College, Clinton, New York.
HIBBARD, DR. HOPE, Department of Zoology, Oberlin College, Oberlin, Ohio.
HILL, DR. SAMUEL, 135 Brunswick Road, Troy, New York.
HINRICHS, DR. MARIE, Women's Gym, University of Illinois, Urbana, Illinois.
HISAW, DR. F. L., Harvard University, Cambridge, Massachusetts.
HOADLEY, DR. LEIGH, Harvard University Biological Laboratories, Cambridge, Massachusetts.
HÖBER, DR. RUDOLF, 5037 Larchwood Avenue, Philadelphia 43, Pennsylvania.
HODES, DR. ROBERT, Tulane University School of Medicine, New Orleans, Louisiana.
HODGE, DR. CHARLES, IV, Temple University, Department of Zoology, Philadelphia, Pennsylvania.
HOGUE, DR. MARY J., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
HOLLAENDER, DR. ALEXANDER, P. O. Box W., Clinton Laboratories, Oak Ridge, Tennessee.
HOPKINS, DR. DWIGHT L., Univ. of Illinois, Pier Branch, Navy Pier, Division of Biological Sciences, Chicago, Illinois.
HOPKINS, DR. HOYT S., New York University College of Dentistry, New York City, New York.
HUNTER, DR. FRANCIS R., Department of Physiology, Florida State University, Tallahassee, Florida.
HUTCHENS, DR. JOHN O., Dept. of Physiology, University of Chicago, Chicago 37, Illinois.
HYMAN, DR. LIBBIE H., American Museum of Natural History, New York City, New York.
IRVING, DR. LAURENCE, U. S. Public Health Service, Anchorage, Alaska.
ISELIN, MR. COLUMBUS O'D., Woods Hole, Massachusetts.
JACOBS, DR. M. H., School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.
JENKINS, DR. GEORGE B., 5339–42 Street, N. W., Washington 15, D. C.
JOHLIN, DR. J. M., Vanderbilt University Medical School, Nashville, Tennessee.
JONES, DR. E. RUFFIN, JR., Biology Department, University of Florida, Gainesville, Florida.
KAAN, DR. HELEN W., National Heart Inst., National Institutes of Health, Bethesda 14, Maryland.
KABAT, DR. E. A., Neurological Inst., College of Physicians and Surgeons, New York City, N. Y.

KARUSH, DR. FRED, Dept. of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania.
KAUFMAN, DR. B. P., Carnegie Institute, Cold Spring Harbor, Long Island, New York.
KEMPTON, DR. RUDOLF T., Vassar College, Poughkeepsie, New York.
KETCHUM, DR. BOSTWICK, Woods Hole Oceanographic Inst., Woods Hole, Mass.
KILLE, DR. FRANK R., Carleton College, Northfield, Minnesota.
KIND, DR. C. ALBERT, Department of Chemistry, University of Connecticut, Storrs, Connecticut.
KINDRED, DR. J. E., University of Virginia, Charlottesville, Virginia.
KING, DR. ROBERT L., State University of Iowa, Iowa City, Iowa.
KISCH, DR. BRUNO, 845 West End Ave., New York City, N. Y.
KLEINHOLZ, DR. LEWIS H., Department of Biology, Reed College, Portland, Oregon.
KLOTZ, DR. I. M., Department of Chemistry, Northwestern University, Evanston, Illinois.
KNOWLTON, DR. F. P., 1356 Westmoreland Avenue, Syracuse, New York.
KOPAC, DR. M. J., New York University, Washington Square College, New York City, New York.
KRAHL, DR. M. E., Washington University School of Medicine, Dept. of Biological Chemistry, St. Louis, Mo.
KREIG, DR. WENDELL J. S., 303 East Chicago Avenue, Chicago, Illinois.
KUFFLER, DR. STEPHEN, Dept. of Ophthalmology, Johns Hopkins Hospital, Baltimore 5, Maryland.
KUNITZ, DR. MOSES, Rockefeller Institute, Princeton, New Jersey.
LACKEY, DR. JAMES B., Univ. of Florida, Dept. Civil Engineering, Gainesville, Fla.
LANCEFIELD, DR. D. E., Queens College, Flushing, New York.
LANCEFIELD, DR. REBECCA C., Rockefeller Inst., 66th St. and York Ave., New York City, New York.
LANDIS, DR. E. M., Harvard Medical School, Boston 15, Massachusetts.
LANGE, DR. MATHILDA M., Box 307, Central Valley, New York.
LANSING, DR. ALBERT I., Department of Anatomy, Washington University, St. Louis 10, Missouri.
LAVIN, DR. GEORGE I., Rockefeller Inst. for Medical Research, New York City, New York.
LAZAROW, DR. ARNOLD, Western Reserve University School of Medicine, Cleveland 6, Ohio.
LEE, DR. RICHARD E., Cornell University College of Medicine, New York City, New York.
LEFEVRE, DR. PAUL G., Division of Biology & Medicine, U. S. Atomic Energy Commission, Washington 25, D. C.
LESSLER, DR. MILTON L., Department of Physiology, Ohio State University, Columbus, Ohio.
LEVY, DR. MILTON, Chemistry Dept., New York University School of Medicine, New York City, N. Y.
LEWIS, DR. I. F., University of Virginia, Charlottesville, Virginia.
LITTLE, DR. E. P., 828 Washington Ave., Fairborn, Ohio.

LOCHHEAD, DR. JOHN H., Department of Zoology, University of Vermont, Burlington, Vermont.
LOEB, DR. LEO, 40 Crestwood Drive, St. Louis 5, Missouri.
LOEB, DR. R. F., 180 Fort Washington Avenue, New York City, New York.
LOEWI, DR. OTTO, 155 East 93rd Street, New York City, New York.
LOVE, DR. LOIS H., 4253 Regent Street, Philadelphia 4, Pennsylvania.
LUCKÉ, DR. BALDUIN, University of Pennsylvania, Philadelphia 4, Pennsylvania.
LYNCH, DR. CLARA J., Rockefeller Inst., 66th Street and York Avenue, New York City, New York.
LYNCH, DR. RUTH STOCKING, Dept. of Botany, University of California, Los Angeles 24, California.
LYNN, DR. WILLIAM G., Dept. Biology, Catholic University of America, Washington, D. C.
MACDOUGAL, DR. MARY S., Mt. Vernon Apts., 423 Clairmont Avenue, Decatur, Georgia.
MCCOUCH, DR. MARGARET SUMWALT, University of Pennsylvania Medical School, Philadelphia, Penn.
MCDONALD, SISTER ELIZABETH SETON, Dept. of Biology, College of Mt. St. Joseph, Mt. St. Joseph, Ohio.
MCDONALD, DR. MARGARET H., Carnegie Inst. of Washington, Cold Spring Harbor, Long Island, N. Y.
MCGREGOR, DR. J. H., Columbia University, New York City, New York.
MACKLIN, DR. CHARLES C., School of Medicine, University of Western Ontario, London, Canada.
MAGRUDER, DR. SAMUEL R., Dept. of Anatomy, Tufts Medical School, 136 Harrison Ave., Boston, Mass.
MALONE, DR. E. F., 153 Cortland Avenue, Winter Park, Florida.
MANWELL, DR. REGINALD D., Syracuse University, Syracuse, New York.
MARMONT, DR. GEORGE H., 4408 Via Azalea, Palos Verdes Estates, Los Angeles, California.
MARSHAK, DR. A., 32 Orange Street, Brooklyn 2, New York.
MARSLAND, DR. DOUGLAS A., Washington Square College, New York University, New York City, N. Y.
MARTIN, DR. EARL A., Department of Biology, Brooklyn College, Brooklyn, New York.
MATHEWS, DR. A. P., Woods Hole, Massachusetts.
MATTHEWS, DR. SAMUEL A., Thompson Biological Laboratory, Williams College, Williamstown, Mass.
MAYOR, DR. JAMES W., 8 Gracewood Park, Cambridge 58, Massachusetts.
MAZIA, DR. DANIEL, University of California, Department of Zoology, Berkeley 4, California.
MEDES, DR. GRACE, Lankenau Research Institute, Philadelphia, Pennsylvania.
MEIGS, MRS. E. B., 1736 M Street N.W., Washington, D. C.
MEMHARD, MR. A. R., Riverside, Connecticut.
MENKIN, DR. VALY, Dept. of Surgical Research, Temple University Medical School, Philadelphia, Penn.
METZ, DR. C. B., Dept. of Zoology, University of North Carolina, Chapel Hill, N. C.

METZ, DR. CHARLES W., University of Pennsylvania, Philadelphia, Pennsylvania.
MILLER, DR. J. A., Basic Science Building, Emory University, Georgia.
MILNE, DR. LORUS J., Dept. of Zoology, University of New Hampshire, Durham, New Hampshire.
MINNICH, DR. D. E., Dept. of Zoology, University of Minnesota, Minneapolis 14, Minnesota.
MITCHELL, DR. PHILIP H., 1414 Grove Terrace, Winter Park, Florida.
MOE, MR. HENRY A., Secretary General, Guggenheim Memorial Foundation, 551 Fifth Ave., New York 17, New York.
MOORE, DR. CARL R., University of Chicago, Chicago 37, Illinois.
MOORE, DR. GEORGE M., Department of Zoology, University of New Hampshire, Durham, N. H.
MOUL, DR. E. T., Dept. of Botany, Rutgers University, New Brunswick, New Jersey.
MOUNTAIN, MRS. J. D., 9 Coolidge Avenue, White Plains, New York.
MULLER, DR. H. J., Department of Zoology, Indiana University, Bloomington, Indiana.
NABRIT, DR. S. M., Atlanta University, Morehouse College, Atlanta, Georgia.
NACHMANSOHN, DR. D., College of Physicians and Surgeons, New York City, New York.
NAVEZ, DR. ALBERT E., 206 Churchill's Lane, Milton 86, Massachusetts.
NEUBERG, DR. CARL, 11 E. 74th Street, New York 21, New York.
NEURATH, DR. H., Dept. of Biochemistry, University of Washington, Seattle 5, Washington.
NEWMAN, DR. H. H., 173 Devon Drive, Clearwater, Florida.
NICOLL, DR. PAUL A., Department of Physiology, Indiana University, Bloomington, Indiana.
NICHOLS, DR. M. LOUISE, Rosemont, Pennsylvania.
NORTHROP, DR. JOHN H., Dept. of Bacteriology, University of California, Berkeley 4, California.
OCHOA, DR. SEVERO, New York University College of Medicine, New York 16, New York.
OPPENHEIMER, DR. JANE M., Dept. of Biology, Bryn Mawr College, Bryn Mawr, Pennsylvania.
OSBURN, DR. R. C., Botany-Ecology Bldg., Ohio State University, Columbus 10, Ohio.
OSTER, DR. ROBERT H., University of Maryland School of Medicine, Baltimore 1, Maryland.
OSTERHOUT, DR. W. J. V., Rockefeller Inst., 66th Street and York Avenue, New York City, N. Y.
OSTERHOUT, MRS. MARION IRWIN, Rockefeller Inst., 66th St. and York Ave., New York City, N. Y.
PACKARD, DR. CHARLES, Woods Hole, Massachusetts.
PAGE, DR. IRVINE H., Cleveland Clinic, Cleveland, Ohio.
PAPPENHEIMER, DR. A. M., 45 Holden Street, Cambridge, Massachusetts.
PARKER, DR. G. H., Harvard University, Cambridge, Massachusetts.
PARMENTER, DR. CHARLES L., Dept. of Zoology, University of Pennsylvania, Philadelphia, Penn.

PARPART, DR. ARTHUR K., Department of Biology, Princeton University, Princeton, New Jersey.
PATTEN, DR. BRADLEY M., University of Michigan Medical School, Ann Arbor Michigan.
PEEBLES, DR. FLORENCE, 380 Rosemont Avenue, Pasadena 3, California.
PERKINS, DR. JOHN F., Dept. of Physiology, University of Chicago, Chicago 37, Illinois.
PETTIBONE, DR. MARIAN H., U. S. Nat. Museum, Div. of Marine Invertebrates, Washington 16, D. C.
PIERCE, DR. MADELENE E., Vassar College, Poughkeepsie, New York.
PLOUGH, DR. HAROLD H., Amherst College, Amherst, Massachusetts.
POLLISTER, DR. A. W., Columbia University, New York City, New York.
POND, DR. SAMUEL E., 53 Alexander Street, Manchester, Connecticut.
PRATT, DR. FREDERICK H., 105 Hundreds Road, Wellesley Hills 82, Massachusetts.
PROSSER, DR. C. LADD, 401 Natural History Bldg., University of Illinois, Urbana, Illinois.
PROVASOLI, DR. LUIGI, Dept. of Biology, Haskins Laboratory, 305 E. 43 St., New York 17, New York.
QUASTEL, DR. JUDA H., Department of Biochemistry, McGill University, Montreal, Canada.
RAMSEY, DR. ROBERT W., University of Virginia Medical School, Richmond, Virginia.
RAND, DR. HERBERT W., 7 Siders Pond Road, Falmouth, Massachusetts.
RANKIN, DR. JOHN S., Department of Zoology, University of Connecticut, Storrs, Connecticut.
REDFIELD, DR. ALFRED C., Woods Hole, Massachusetts.
REID, DR. W. M., Monmouth College, Monmouth, Illinois.
REINER, DR. J. M., Columbia-Presbyterian Medical Center, 622 West 168 St., New York 32, N. Y.
RENN, DR. CHARLES E., 200 Whitehead Hall, Johns Hopkins University, Baltimore, Maryland.
REZNIKOFF, DR. PAUL, Cornell University Medical College, 1300 York Avenue, New York City, N. Y.
RICE, DR. E. L., 2241 Seneca Avenue, Alliance, Ohio.
RICHARDS, DR. A., 2950E Mabel Street, Tucson, Arizona.
RICHARDS, DR. A. GLENN, Entomology Dept., University Farm, University of Minnesota, St. Paul, Minnesota.
RICHARDS, DR. OSCAR W., American Optical Company, P. O. Box 1377, Stamford, Connecticut.
RIESER, DR. PETER, Department of Physiology, University of Iowa, Iowa City, Iowa.
RIGGS, MR. LAWRASON, 120 Broadway, New York City, New York.
ROGICK, DR. MARY D., College of New Rochelle, New Rochelle, New York.
ROMER, DR. ALFRED S., Harvard University, Museum of Comparative Zoology, Cambridge, Mass.
RONKIN, DR. RAPHAEL R., Department of Physiology, University of Delaware, Newark, Delaware.

ROOT, DR. R. W., Department of Biology, College of the City of New York, New York City, N. Y.

ROOT, DR. W. C., Columbia University, College of Physicians and Surgeons, Dept. of Physiology, New York City, New York.

ROSE, DR. S. MERYL, Department of Zoology, University of Illinois, Champaign, Illinois.

ROSENTHAL, DR. THEODORE B., Washington University, St. Louis 10, Missouri.

ROSSI, DR. HAROLD H., Department of Radiology, Columbia University, New York 32, New York.

ROTH, DR. JAY S., Department of Biochemistry, Hahnemann Medical College, Philadelphia 2, Penn.

ROTHENBERG, DR. M. A., Chief, Biochemistry Branch, C. W. Division, Duguay Proving Ground, Tooele, Utah.

RUGH, DR. ROBERTS, Radiological Research Laboratory, College of Physicians and Surgeons, New York City, New York.

RUNNSTRÖM, DR. JOHN, Wenner-Grens Institute, Stockholm, Sweden.

RYAN, DR. FRANCIS J., Columbia University, New York City, New York.

SAMPSON, DR. MYRA M., Smith College, Northampton, Massachusetts.

SANDEEN, DR. MURIEL I., Department of Zoology, Duke University, Durham, North Carolina.

SAUNDERS, MR. LAWRENCE, R. D. 7, Bryn Mawr, Pennsylvania.

SCHAEFFER, DR. ASA A., Biology Department, Temple University, Philadelphia, Pennsylvania.

SCHARRER, DR. ERNST A., Univ. of Colorado, Dept. of Anatomy, School of Medicine and Hospital, 4200 E. 9th Avenue, Denver 7, Colorado.

SCHECHTER, DR. VICTOR, College of the City of New York, New York City, New York.

SCHMIDT, DR. L. H., Christ Hospital, Cincinnati, Ohio.

SCHMITT, DR. FRANCIS O., Dept. of Biology, Massachusetts Inst. of Technology, Cambridge, Mass.

SCHMITT, DR. O. H., Physics Department, University of Minnesota, Minneapolis 14, Minnesota.

SCHOLANDER, DR. P. F., Woods Hole, Massachusetts.

SCHOTTÉ, DR. OSCAR E., Department of Biology, Amherst College, Amherst, Massachusetts.

SCHRADER, DR. FRANZ, Department of Zoology, Columbia University, New York City, New York.

SCHRADER, DR. SALLY HUGHES, Dept. of Zoology, Columbia University, New York City, New York.

SCHRAMM, DR. J. R., University of Pennsylvania, Philadelphia, Pennsylvania.

SCOTT, DR. ALLAN C., Colby College, Waterville, Maine.

SCOTT, SISTER FLORENCE M., Seton Hill College, Greensburg, Pennsylvania.

SCOTT, DR. GEORGE T., Oberlin College, Oberlin, Ohio.

SEARS, DR. MARY, Woods Hole Oceanographic Inst., Woods Hole, Mass.

SEMPLE, MRS. R. BOWLING, 1767 P Street, N.W., Washington, D. C.

SEVERINGHAUS, DR. AURA E., Dept. of Anatomy, College of Physicians and Surgeons, New York City.

SHANES, DR. ABRAHAM M., Experimental Biology and Medicine Inst., National Institutes of Health, Bethesda 14, Maryland.
SHAPIRO, DR. HERBERT, 5800 N. Camac Street, Philadelphia 41, Pennsylvania.
SHAVER, DR. JOHN R., Department of Zoology, University of Missouri, Columbia, Missouri.
SHUMWAY, DR. WALDO, Stevens Institute of Technology, Hoboken, New Jersey.
SICHEL, DR. FERDINAND J. M., University of Vermont, Burlington, Vermont.
SICHEL, MRS. F. J. M., 35 Henderson Terrace, Burlington, Vermont.
SLIFER, DR. ELEANOR H., Department of Zoology, State University of Iowa, Iowa City, Iowa.
SMITH, DR. DIETRICH CONRAD, Dept. of Physiology, Univ. of Maryland School of Medicine, Baltimore, Maryland.
SMITH, DR. EDWARD H., Woods Hole Oceanographic Institution, Woods Hole, Massachusetts.
SMITH, MR. HOMER P., General Manager, Marine Biological Laboratory, Woods Hole, Mass.
SMITH, DR. RALPH I., Department of Zoology, University of California, Berkeley 4, California.
SONNEBORN, DR. T. M., Department of Zoology, Indiana University, Bloomington, Indiana.
SONNENBLICK, DR. B. P., 40 Rector Street, Newark 2, New Jersey.
SPEIDEL, DR. CARL C., University of Virginia, University, Virginia.
SPRATT, DR. NELSON T., Dept. of Zoology, University of Minnesota, Minneapolis 14, Minnesota.
STEINBACH, DR. HENRY BURR, University of Minnesota, Zoology Dept., Minneapolis 14, Minnesota.
STERN, DR. KURT G., Polytechnic Inst., Dept. of Chemistry, 84 Livingston St., Brookyln, N. Y.
STEWART, DR. DOROTHY, Rockford College, Rockford, Illinois.
STOKEY, DR. ALMA G., Department of Botany, Mt. Holyoke College, South Hadley, Mass.
STRAUS, DR. W. L., Johns Hopkins Medical School, Baltimore 5, Maryland.
STUNKARD, DR. HORACE W., New York University, New York City, New York.
STURTEVANT, DR. ALFRED H., California Institute of Technology, Pasadena 4, California.
SWOPE, MR. GERARD, JR., 570 Lexington Avenue, New York 22, New York.
SZENT-GYORGYI, DR. A. E., Woods Hole, Massachusetts.
SZENT-GYORGYI, DR. ANDREW G., Inst. for Muscle Research, Woods Hole, Mass.
TASHIRO, DR. SHIRO, University of Cincinnati Medical College, Cincinnati, Ohio.
TAYLOR, DR. WILLIAM RANDOLPH, University of Michigan, Ann Arbor, Michigan.
TEWINKEL, DR. LOIS E., Dept. of Zoology, Smith College, Northampton, Massachusetts.
TRACY, DR. HENRY C., Department of Anatomy, University of Mississippi, University, Miss.
TRAGER, DR. WILLIAM, Rockefeller Inst., 66th Street and York Avenue, New York 21, New York.
TRINKAUS, DR. J. PHILIP, Department of Zoology, Osborn Zoological Laboratory, New Haven, Conn.

TURNER, DR. C. L., Northwestern University, Evanston, Illinois.
TYLER, DR. ALBERT, California Institute of Technology, Pasadena, California.
UHLENHUTH, DR. EDWARD, University of Maryland School of Medicine, Baltimore, Maryland.
VILLEE, DR. CLAUDE A., Harvard Medical School, Boston 15, Massachusetts.
WAINIO, DR. W. W., Bureau of Biological Research, Rutgers University, New Brunswick, N. J.
WALD, DR. GEORGE, Biological Laboratory, Harvard University, Cambridge, Massachusetts.
WARBASSE, DR. JAMES P., Woods Hole, Massachusetts.
WARNER, DR. ROBERT C., Dept. of Chemistry, New York Univ. College of Medicine, New York 16, N. Y.
WARREN, DR. HERBERT S., 610 Montgomery Avenue, Bryn Mawr, Pennsylvania.
WATERMAN, DR. T. H., Osborn Zoological Laboratory, Yale University, New Haven, Connecticut.
WEBB, DR. H. MARGUERITE, Department of Physiology and Bacteriology, Goucher College, Towson, Md.
WEISS, DR. PAUL A., Department of Zoology, University of Chicago, Chicago 37, Illinois.
WENRICH, DR. D. H., University of Pennsylvania, Philadelphia, Pennsylvania.
WHEDON, DR. A. D., 21 Laroncrest, Danbury, Connecticut.
WHITAKER, DR. DOUGLAS M., P. O. Box 2514, Stanford University, California.
WHITE, DR. E. GRACE, Wilson College, Chambersburg, Pennsylvania.
WHITING, DR. ANNA R., University of Pennsylvania, Philadelphia, Pennsylvania.
WHITING, DR. PHINEAS W., Zoological Lab., University of Pennsylvania, Philadelphia, Penn.
WICHTERMAN, DR. RALPH, Biology Department, Temple University, Philadelphia, Pennsylvania.
WIEMAN, DR. H. L., University of Cincinnati, Cincinnati, Ohio.
WILBER, DR. C. G., Medical Laboratories, Applied Physiology Branch, Army Chemical Center, Md.
WILLIER, DR. B. H., Department of Biology, Johns Hopkins University, Baltimore, Maryland.
WILSON, DR. J. W., Brown University, Providence, Rhode Island.
WILSON, DR. WALTER L., Department of Physiology, University of Vermont College of Medicine, Burlington, Vermont.
WITSCHI, DR. EMIL, Department of Zoology, State University of Iowa, Iowa City, Iowa.
WOLF, DR. ERNST, Pendleton Hall, Wellesley College, Wellesley, Massachusetts.
WOOD, DR. RICHARD D., Department of Botany, Rhode Island State College, Kingston, Rhode Island.
WOODWARD, DR. ALVALYN E., Department of Zoology, University of Michigan, Ann Arbor, Michigan.
WOODWARD, DR. ARTHUR A., Department of Zoology, Washington University, St. Louis 5, Missouri.
WRIGHT, DR. PAUL A., Department of Zoology, University of Michigan, Ann Arbor, Michigan.
WRINCH, DR. DOROTHY, Smith College, Northampton, Massachusetts.

YNTEMA, DR. C. L., Department of Anatomy, State University of New York College of Medicine, Syracuse 10, New York.
YOUNG, DR. B. P., Cornell University, Ithaca, New York.
YOUNG, DR. D. B., 7128 Hampden Lane, Bethesda 14, Maryland.
YUDKIN, DR. WARREN H., Dept. of Chemistry, Northwestern University, Evanston, Illinois.
ZINN, DR. DONALD J., Box 66, State College, Kingston, Rhode Island.
ZORZOLI, DR. ANITA, Dept. of Physiology, Southern Illinois University, Carbondale, Illinois.
ZWILLING, DR. E., Department of Genetics, University of Connecticut, Storrs, Connecticut.

### 3. ASSOCIATES OF THE MARINE BIOLOGICAL LABORATORY

ADLER, MRS. CYRUS
ADLER, DR. AND MRS. F. H.
ALLEN, MR. AND MRS. EUGENE Y.
BARTOW, MRS. FRANCIS D.
BEHNKE, MR. JOHN A.
BELL, MRS. ARTHUR
BROWN, MRS. SARAH MEIGS
BROWN, MRS. THEODORE E.
BUFFINGTON, MR. AND MRS. GEORGE
BURK, DR. AND MRS. DEAN
CAHOON, MR. AND MRS. SAM
CALKINS, MRS. GARY N.
CLAFF, MRS. C. LLOYD
CLARK, MR. ALFRED HULL
CLOWES, MRS. G. H. A.
COOPER, MR. AND MRS. CHARLES P.
CRANE, MRS. FRANCES A.
CRANE, MRS. MURRAY
CRANE, MR. RICHARD
CROSSLEY, MR. AND MRS. ARCHIBALD
CROWELL, MR. PRINCE S.
CURTIS, DR. AND MRS. W. D.
DANIELS, MR. AND MRS. HAROLD
DRAPER, MRS. MARY C.
DRINKER, DR. AND MRS. CECIL
ELSMITH, MRS. DOROTHY
ENDERS, MR. FRED
FAY, MR. HENRY H.
FISHER, MRS. BRUCE CRANE
FROST, MRS. FRANK
GANNETT, MR. AND MRS. ROBERT T.
GARFIELD, MRS. I. MCD.
GIFFORD, MR. AND MRS. JOHN A.
GRANT, MRS. MARJORIE S. M.
GREENE, MISS GLADYS M.
HALL, MR. AND MRS. HENRY
HOWE, MRS. HARRISON
HUNT, MRS. REID
JANNEY, MRS. WALTER C.
JEWETT, MR. GEORGE F.
KEEP, MR. AND MRS. FREDERIC A.
KEITH, MR. AND MRS. HAROLD C.
KIDDER, MR. AND MRS. HENRY M.
KNOWER, MRS. HENRY
KOLLER, MRS. LEWIS
LILLIE, MRS. FRANK R.
LILLIE, MRS. RALPH S.
MARVIN, MRS. A. H.
MATHER, DR. AND MRS. FRANK J.
MEIGS, MRS. EDWARD B.
MITCHELL, MRS. JAMES MCC.
MIXTER, MRS. JASON
MONTGOMERY, MRS. T. H.
MOORE, MRS. WILLIAM A.
MORGAN, MRS. T. H.
MORRISON, MR. DAVID
MOTLEY, MRS. THOMAS
MURPHY, DR. WALTER J.
NEWTON, MISS HELEN
NIMS, MR. AND MRS. E. D.
NORMAN, MR. AND MRS. EDWARD
OPPENHEIM-ERRER, DR. AND MRS. PAUL
OSTER, MRS. R. H.
PACKARD, DR. AND MRS. L. B.
PARK, MR. MALCOLM
RATCLIFFE, MRS. THOMAS G.
REDFIELD, MRS. ALFRED
RENTSCHLER, MR. AND MRS. GEORGE
REZNIKOFF, MRS. PAUL

Richards, Mrs. A. N.
Riggs, Mrs. Lawrason
Rudd, Mrs. H. W. Dwight
Sands, Mrs. Adelaide
Saunders, Mrs. Lawrence
Spackman, Miss Emily S.
Spivack, Mr. Leo H.
Springer, Mr. and Mrs. Otto
Steel, Mr. Richard
Stockard, Mrs. Charles R.
Strong, Miss Jane
Swift, Mrs. Katherine W.
Swope, Mr. Gerard
Szent-Györgyi, Mrs. Albert
Tebbetts, Mr. and Mrs. Walter
Ward, Mr. and Mrs. Francis T.
Webster, Mrs. Edwin S.
Wick, Mrs. Myron A.
Wickersham, Mr. and Mrs. James H.
Williston, Professor Samuel
Wilson, Mrs. Edmund B.
Wolfinsohn, Mrs. Wolfe

# STRUCTURE AND FUNCTION IN THE PYLORIC CAECA OF ASTERIAS FORBESI

JOHN MAXWELL ANDERSON

*Arnold Biological Laboratory, Brown University, and Department of Zoology, Cornell University,*[1] *Ithaca, N. Y.*

In his book, *Comparative Physiology*, Scheer (1948, p. 352) makes the following statement concerning Echinodermata: "The process of digestion is not well known. Large diverticula are present in the asteroids. . . . Extracellular digestion is evident . . ., but the function of the extensive diverticula remains unsolved." A review of the literature on this subject reveals that this statement is largely justified. Although repeated attempts have been made to elucidate the mechanisms of digestion and absorption in asteroids, the conclusions drawn have been highly contradictory, and in many cases the experimental procedures are open to serious question. Indeed, it appears that no general agreement has been reached in descriptions of the histological details of the alimentary canal in starfishes.

The present report is a preliminary account of studies undertaken to clear up some of the existing confusion in the histology of the pyloric caeca, and to correlate by means of histochemical techniques the structural and functional aspects of the cells comprising the lining epithelium of these organs. Although no attempt has been made to present here an exhaustive review of all previous studies on the pyloric caeca, a brief analysis of the opinions of other investigators is included and an effort has been made to synthesize from all reliable sources a picture of the probable role of the pyloric caeca in alimentation.

## Material and Methods

Animals used in these investigations were vigorous adult specimens of *Asterias forbesi*, ranging in size from 10 to 20 cm. in diameter. They were obtained by express from Woods Hole, maintained in 50-gallon marine aquaria, and fed regularly with portions of clam and mussel. For studies of the effect of nutritional state on the condition of the digestive diverticula, a series of animals was isolated and starved for varying periods ranging up to 8 weeks. Six to 8 weeks of starvation appeared to be the maximum which an animal of this size could withstand.

For use, one arm was removed from an animal and its paired pyloric caeca dissected out in sea water. Small pieces of the caeca were then taken and fixed in a series of fluids. These samples were subsequently processed and imbedded as appropriate for the various histological and histochemical techniques to be applied, as follows:

1) For general orientation, cell structure, and secretion granules, tissues were fixed in Zenker-formal, imbedded in paraffin by the dioxan method, and sec-

[1] Present address.

tioned at 4 $\mu$. These sections were stained in Mallory's phosphotungstic acid hematoxylin, Heidenhain's iron alum hematoxylin, Harris hematoxylin, or neutral gentian.

2) For demonstration of glycogen and related compounds, fixation was in Helly's fluid or in cold Rossmann, followed by treatment of paraffin sections by the Hotchkiss-McManus periodic acid-Schiff routine. Control slides exposed to the action of a buffered solution of malt diastase were used for differentiation between glycogen and other Schiff-positive substances.

3) For the recognition of mucin and other similar compounds (acid polysaccharides), paraffin sections of tissues fixed in buffered Orth's fluid or in basic lead acetate-formaldehyde were stained overnight in very dilute aqueous solutions of toluidine blue and dehydrated through 95% and absolute alcohol. This routine brings about a metachromatic staining of acid-polysaccharide elements. Representative sections of this material were also treated by the periodic acid-Schiff method.

4) For the localization, and, in part, the characterization of lipids, tissues were fixed in Baker's formal-calcium or formal-saline. For general recognition of lipids, tissues fixed in formal-saline, and soaked at 60° C. for 24 hours in 5% potassium dichromate, were imbedded in gelatin, sectioned on the freezing microtome, colored with Sudan black, and counterstained with Mayer's carmalum. For the localization of triglycerides, similarly fixed, postchromed, imbedded, and sectioned material was stained in 1% Nile blue A and differentiated in 1% acetic acid (Cain, 1947a). Phospholipid was investigated in material fixed in formal-calcium and treated by Baker's acid-hematein method, controlled by the pyridine-extraction test applied to adjacent samples fixed in weak Bouin's fluid (Baker, 1946).

5) For the demonstration of alkaline phosphatase activity, tissues were fixed in ice-cold acetone and imbedded in paraffin. Four-micron sections were incubated at 37° C. in a solution of sodium-beta-glycerophosphate and then carried through the routine of Gomori, in which sites of phosphatase activity are visualized as brown or black deposits of cobalt sulfide. Control sections were processed simultaneously, omitting only the substrate-incubation step.

It is a pleasure to acknowledge the capable technical assistance of Dorothy T. Clarke, Research Assistant in Biology at Brown University, in the conduct of a large part of this work.

## Observations

### *A. General structure of the pyloric caecum*

Each diverticulum constitutes one member of a pair in each arm, representing branches produced by the bifurcation of the single pyloric duct proceeding into the arm from an angle of the pyloric stomach. Each diverticulum is suspended by a pair of longitudinal mesenteries, enclosing between them a part of the epigastric coelom. The sheets comprising the mesenteries are continuous with the somatic mesothelium lining the body wall, and with the splanchnic mesothelium covering the diverticulum.

The pyloric duct continues the length of the caecum, forming a central tubular cavity known as Tiedemann's diverticulum. Serial outpocketings from the lateral walls of this tube form a row of pouches, whose cavities communicate broadly with that of the central passageway. By a series of vertical folds, the walls of these primary pockets form secondary bays, branching in a radial pattern. The secondary bays may also be subdivided, by both vertical and horizontal folds in their walls, to form the ultimate blind cavities of the caecum.

The wall of this complex organ is relatively simple, consisting of layers which may be described as follows:

a. As noted above, the organ is covered by a peritoneal layer. This is composed of small, cuboidal cells and is stretched thin over convexities but thrown into folds in markedly indented areas. Each cell of this layer bears a single, long flagellum.

b. Layers of muscular, nervous, and connective tissue fibers, variously represented, lie under the splanchnic peritoneum (for details, see Hamann, 1885, and Chadwick, 1923).

c. The epithelial lining is chiefly responsible for the thickness of the wall. It is composed of a single layer of extremely tall, slender cells forming a pseudostratified columnar epithelium, resting upon a basement membrane. This layer contains several distinct cell types, described below in detail. Speaking generally, the free ends of the epithelial cells are covered by a thick brush border, and each cell bears a single long flagellum. The height of the epithelium varies tremendously, being least in the angles of evaginated folds in the wall. At these points the lumen appears to extend almost to the basement membrane, while in the walls between these thin spots the epithelium may be from 75 to 90 $\mu$ in height.

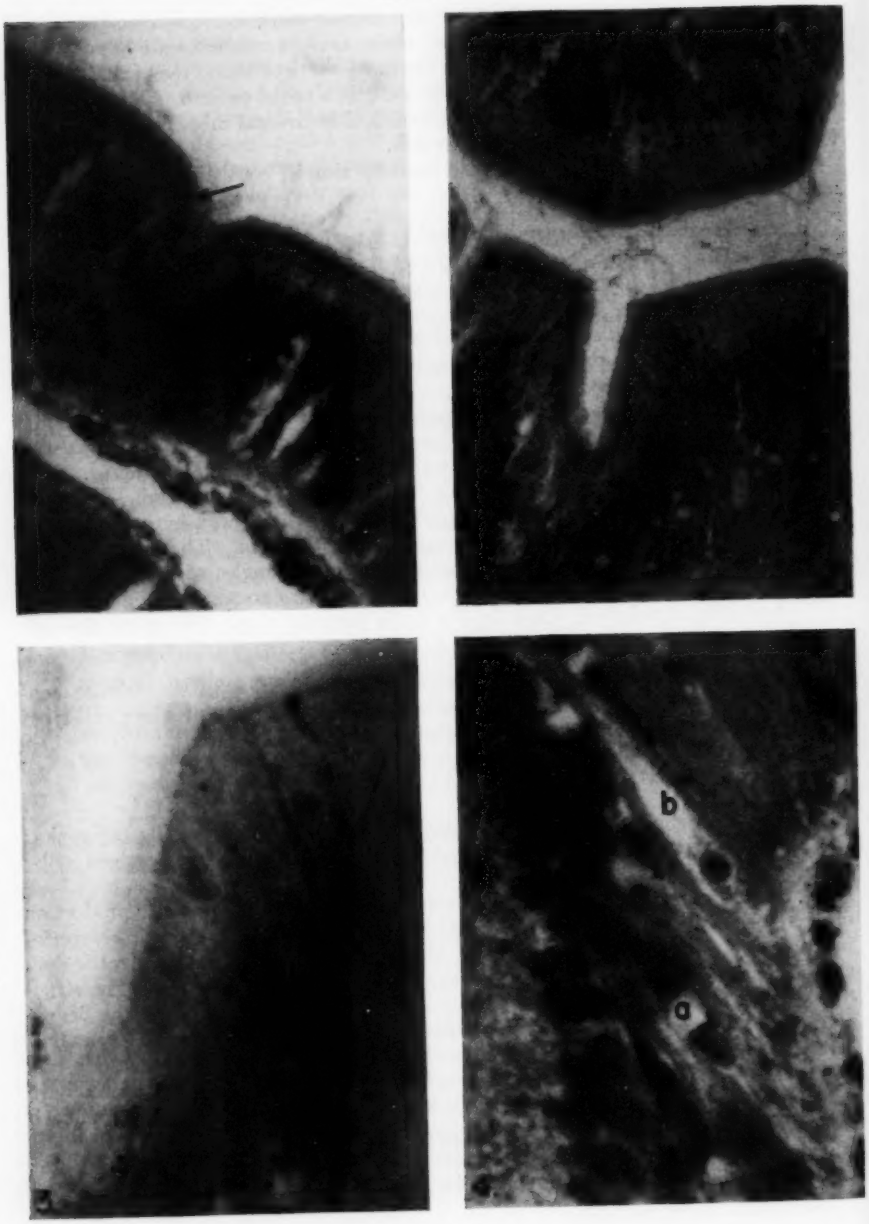
The following section will be concerned with elucidation of the cellular constituents of this lining epithelium and the characterization of the various cell-types represented, in terms of their histological and histochemical details.

*B. Organization of the lining epithelium*

The cells of which the epithelium is composed fall into the following categories: (a) special current-producers; (b) storage cells; (c) secretory or zymogen cells; (d) mucus gland cells.

a. Special current-producers are structurally highly adapted to the production of currents in the lumen of the diverticulum but apparently have no other special function. These cells are characteristic of the lining of Tiedemann's diverticulum and of the oral and aboral walls of the primary pockets arising from it. The structural details of these cells vary with their location, and apparently with the degree of crowding to which they are subjected. In the aboral wall of Tiedemann's diverticulum, between the attachments of the mesenteries, they are moderately tall, remarkably slender, and very closely packed (Fig. 9). In these cells the nuclei are long and spindle-shaped, measuring about 8 $\mu \times 2$ $\mu$. They are granular, and each contains a small nucleolus. In other regions the current-producers are taller, less crowded, with densely-staining oval nuclei. In all, the nuclei lie at various depths in the epithelium, from a point near the basement membrane to within a short

PLATE I



distance of the free edge (Fig. 1). The cells are broadest in the nuclear region, and from the basal end of this area in each cell a tapering fibrous process extends to the basement membrane. Although this is difficult to ascertain, each cell appears to be represented at the surface of the lumen, where it bears a conspicuous brush border and a single long flagellum, ½ to ⅓ as long as the cell. The flagellum springs from a prominent blepharoplast lying just under the brush border and sending downward into the cytoplasm a stout chromophilic strand.

In all areas of which these cells are characteristic, mucus gland cells are abundant (Figs. 1 and 10).

b. Storage cells, in the lateral walls of the primary pockets and throughout the lining of the secondary bays and ultimate branches of the caeca, replace the current-producers as the most numerous class. These cells average about 5 $\mu$ in diameter and vary in height from 15 $\mu$ to a maximum of about 90 $\mu$, depending upon their location and upon the size of the animal. Their nuclei are broadly oval or subspherical and generally stain densely and uniformly, except for the small nucleolus. The nuclei occupy a broad band limited approximately to the middle third of the cells. Each cell bears, again, a single flagellum originating in an apical blepharoplast and extending well beyond the brush border. Sections cut tangentially to the surface of the epithelium in these regions show that the cells are roughly polygonal in cross-section, and that the blepharoplasts, one in each cell, do not occupy the center of the apex but lie very near the cell membrane to one side (Fig. 6) and may be attached to it. Close study of sections passing longitudinally through this region gives the impression that the blepharoplast gives rise to one strand running in contact with the cell membrane and to another passing more directly downward into the cytoplasm (Fig. 3).

In a normal, well-fed animal, these cells contain abundant deposits of lipids, lying both above and below the nucleus in the form of moderate-sized droplets. The lipid droplets are larger and more numerous in the basal portions of the cells; above the nuclear region they become smaller and more sparse and are usually lacking from the distal quarter of the cell (Fig. 7). The Nile blue technique reveals that triglycerides predominate in the constitution of these lipid deposits; no elements have been recognized in the storage cells which are sudanophilic and stain other than pink with Nile blue. The acid hematein test has failed to reveal phospholipid deposits.

---

Figures 1 through 6 represent sections of material fixed in Helly's fluid, sectioned at 4 to 6 $\mu$, and stained in Mallory's phosphotungstic acid hematoxylin. In all cases, the indicated magnification is approximately that of the figure as it appears here.

PLATE I

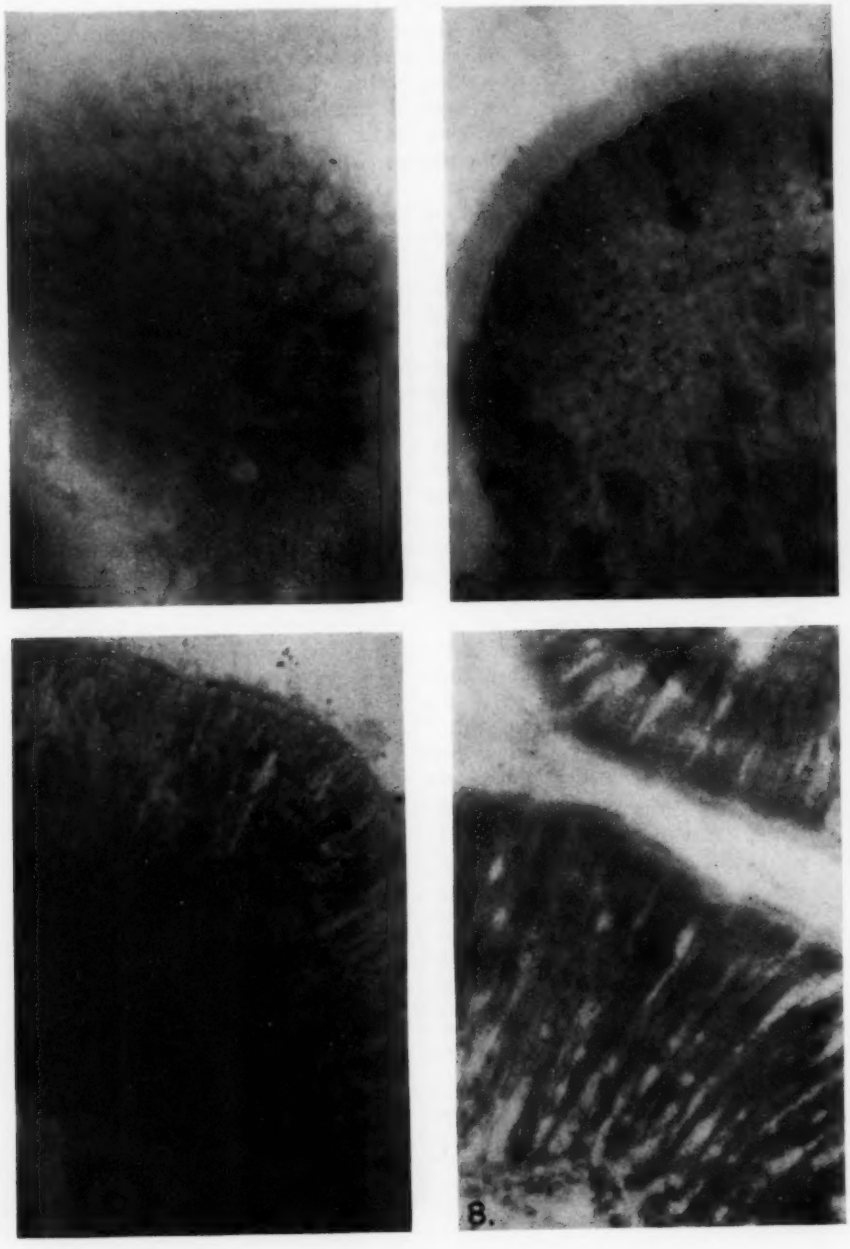
FIGURE 1. Special current-producing cells, moderately crowded region. Lumen above, perivisceral coelom below. Note row of blepharoplasts, brush border, flagella. Large clear areas are mucus goblets; mucus stoma indicated by arrow. 500 ×.

FIGURE 2. Storage and secretory cell areas, from same slide as Figure 1. Note vacuoles surrounded by secretory granules. 500 ×.

FIGURE 3. Part of same region, 1200 ×. Note extent of granule-train indicated by arrow; also divergent chromophilic strands from blepharoplasts.

FIGURE 4. Secretory cell region. At *a*, note relationship between nucleus and secretory products; at *b*, part of a mucus gland cell with empty mucus vacuole. Peritoneum at right; lumen off left. 1200 ×.

PLATE II



The ground-cytoplasm of the storage cells also contains a small amount of glycogen, in addition to more abundant stores of a related compound giving a positive reaction with the Hotchkiss-McManus technique even after prolonged exposure to saliva or malt diastase. This positive reaction is slowly abolished by the action of pancreatin. The substance does not stain metachromatically with toluidine blue. It is presumably a polysaccharide-protein complex. The small deposits of glycogen, and particularly the copious amounts of this unknown compound, have a distribution in the cell similar to that of the lipids; *i.e.*, they are most abundant in the region between nucleus and basement membrane, become scanty above the nucleus, and are absent from the distal ends of the cells (Fig. 9). Storage cells frequently contain large globules, giving the reactions of the unknown substance, scattered in the region above the nucleus (Fig. 9).

In an animal subjected to prolonged starvation, all of these reserves disappear from the storage cells. Glycogen, never abundant, is used first, and its disappearance is followed by a gradual decrease in demonstrable lipids (Fig. 8). The carbohydrate-protein compound also vanishes in the course of 6 to 8 weeks' starvation.

The greenish-yellow color of the pyloric caeca appears to be associated chiefly with the storage cells. Each of these cells contains numerous granules of a greenish pigment, limited to its distal portion. The pigment granules are not sudanophilic but frequently stain after prolonged treatment with phosphotungstic acid hematoxylin.

c. Secretory or zymogen cells are never encountered in the regions occupied by special current-producing cells but are numerous among the storage cells, which they resemble in some respects. Their nuclei are similar in size and shape to those of storage cells but have a more granular, less homogeneous appearance. The nucleus occupies the broadest region of the spindle-shaped cell, and in neighboring cells this swelling may be higher or lower, presenting a staggered arrangement of the nuclei in these closely-packed cells.

The outstanding feature of the secretory cells is their content of secretory granules, presumably zymogenic (Figs. 2, 3 and 4). These granules lie in clumps in the expanded region adjacent to the nucleus, both above and below the nucleus, and may extend in one or more rows completely to the free end of the cell. Very active cells also show dense aggregations of granules in their basal ends. Associated usually with the supranuclear granule clump appears a clear vacuole; very rarely, but occasionally, this vacuole may lie below the nucleus. The granules pass upward through the cell, crowding past the nucleus, and may often be observed as if on the

PLATE II

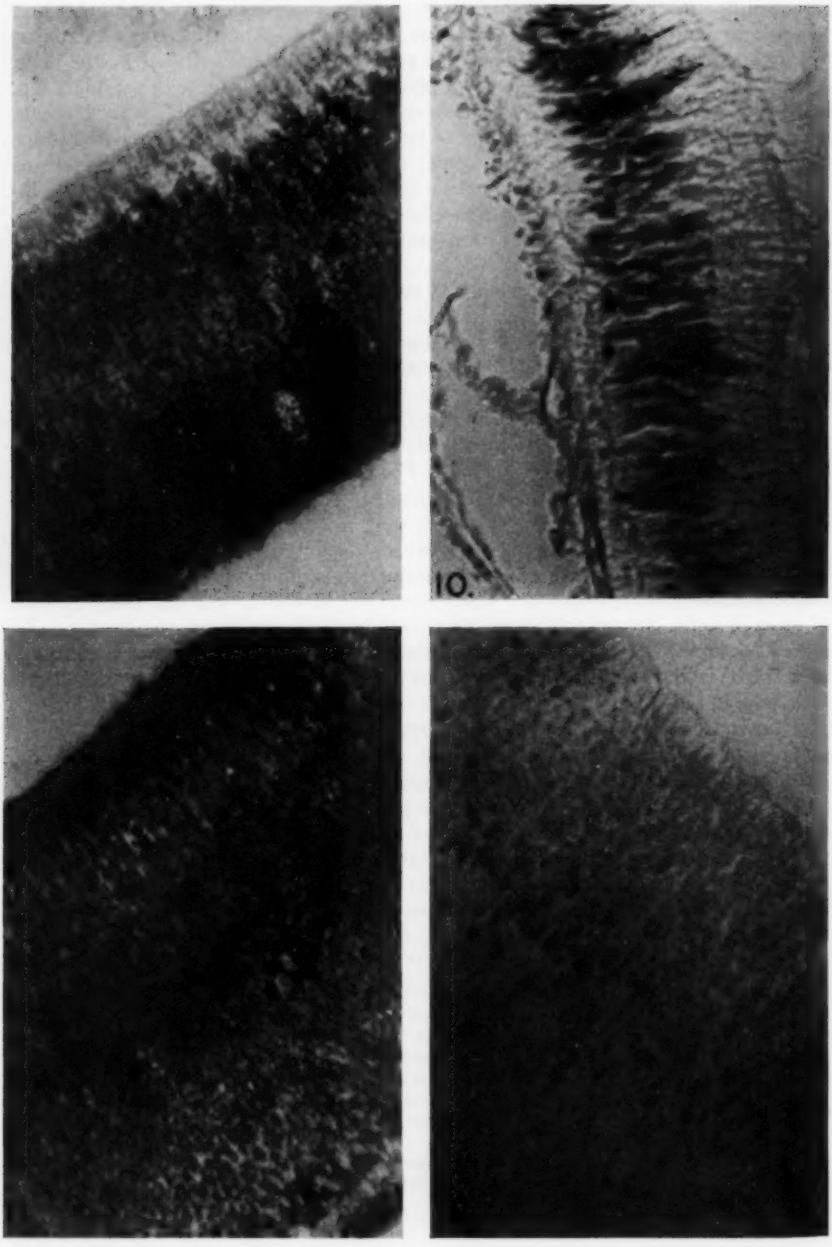
FIGURE 5. Distal ends of storage and secretory cells, section tangential to surface of epithelium. Note shape of cells, eccentric positions of blepharoplasts and their apparent attachment to cell membrane. At *a*, mucus stoma; at *b*, secretory cell. 1500 ×.

FIGURE 6. Distal ends of storage and secretory cells, longitudinal section. Note brush border, blepharoplasts, chromophilic strands, flagella. 1500 ×.

FIGURE 7. Storage cell area, showing lipid deposits. Note concentration of droplets toward basal ends of cells. Formal-saline; frozen section colored with Sudan black, counterstained with carmalum. 500 ×.

FIGURE 8. Storage cell area in animal after 8 weeks' starvation. Preparation exactly as in Figure 7; note exhaustion of stored lipids. 500 ×.

PLATE III



point of being extruded at the free end. The vacuole also appears to move upward through the cell with the granular clump but has not been observed near the apical end.

The vacuoles and secretory granules are well preserved by all effective cytoplasmic fixatives, and the granules are easily demonstrable by such staining methods as Heidenhain's iron alum hematoxylin, Mallory's phosphotungstic acid hematoxylin, and the neutral gentian technique. In frozen sections of material prepared for fat staining, they appear as clear, somewhat refractile spherules. Under the action of fixatives containing acetic acid they break down, and in sections of such material the secretory cells appear to contain only a rather densely granular, basophilic cytoplasm.

To date it has been impossible to establish the morphology of the distal ends of secretory cells. Unless it contains secretory granules the free end of a cell cannot with certainty be related to an underlying granule-filled nuclear region. The crowding of the cells is such that even in thin sections several storage and secretory cells are usually superimposed. In an unmistakable secretory-cell apex, the cytoplasm is so packed with granules as to obscure details (Fig. 5).

d. Mucus gland cells, as indicated above, are most numerous in regions lined by special current-producers. They also occur, less frequently, scattered among the storage and secretory cells. Mucus cells invariably extend almost to the basement membrane of the epithelium, and the nuclei of these cells are basally located. The overlying space is filled with mucus which usually expands the cell to a diameter many times that of the neighboring cells (Figs. 1 and 4). Above this expanded portion, at a variable distance from the base, the cell narrows and finally opens into the lumen of the caecum at a restricted stoma, devoid of any brush border or other structural specialization (Fig. 1). In fact, one may question whether the mucus cell as such comprises anything more than the basal portion of the gland; it is conceivable that the secretion is released from the gland cell at some lower point and exudes through a flask-shaped canal formed by the walls of adjacent cells.

In teased living preparations examined in sea water the secretion of the mucus glands consists of clumps of large globules. In fixed, sectioned preparations the globules are collapsed, and the secretion has the form of films or strands traversing the cavity filled in life by the globules. The material composing these strands reacts vigorously with the Schiff reagent after treatment with periodate and exhibits a strong, purplish metachromasia with dilute toluidine blue (Fig. 10). After its

PLATE III

FIGURE 9. Storage cell area. Rossmann; Hotchkiss-McManus technique. Note basal concentration of Schiff-positive material, most of which is not removed by diastase; empty apical regions; large scattered globules. Coelom below, peritoneum missing. 500 ×.

FIGURE 10. Mucus gland cells in special current-producing area near mesentery attachment; *cf.* Figure 1. Note length of flagella (right), densely crowded cells. Arrows indicate two large metachromatically-staining mucus glands. Basic lead acetate-formaldehyde; dilute toluidine blue. 500 ×.

FIGURE 11. Storage cell area, alkaline phosphatase activity. Note concentration of activity at brush border and in apical areas; *cf.* Figures 10 and 12. Cold acetone; Gomori technique. The carmalum counterstain is responsible for most of the dark appearance of the lower areas. 500 ×.

FIGURE 12. Section adjacent to that shown in Figure 11, treated similarly but incubation with substrate omitted. Nuclei stained with carmalum. 500 ×.

release from the stomata, the secretion spreads thinly and generally over the free ends of the epithelial cells and by its characteristic reactions can be demonstrated about the fibers of the brush border.

In terms of the epithelium as a whole, alkaline phosphatase activity appears to be largely limited to the regions occupied by the storage cells. It is lacking or weak in the areas lined by the current-producing cells. This enzymatic activity is very strong at the free ends of the cells, centering about the region of the brush border and in the distal areas previously seen to be free of stored reserves (Fig. 11). It is weak or lacking in the deeper portions of the epithelium.

## Discussion

It comes as a surprise to discover that both inner and outer epithelia of the alimentary canal are provided with vibratile organelles which from their length and distribution must properly be termed flagella, instead of cilia. Most previous investigators in describing these epithelia have referred to them as ciliated. For example, Hamann (1885) cites *"die heftige Wimperung"* of the caeca, and (p. 62) specifically likens the points of attachment of these organelles to those of the cilia on the molluscan gill. Van der Heyde in both the English (1922) and French (1923) versions of his dissertation speaks of cilia, as does Chadwick (1923). In justice to Hamann it should be stated that his observations of the current-producing organelles were largely accurate; he writes (p. 62) (here in connection with the cardiac stomach): *"Die Epithelzellen des Magendarmes sind lange cylindrisch bis haarfeine Zellen, welche je eine Wimper tragen."* Correct as to number, his use of the word *Wimper* is not in accordance with modern usage. On the other hand, Schneider (1902) describing the same elements uses the word *Geissel*, which in translation would be more likely to appear as "flagellum" than would *Wimper*. Authors concerned more with the physiology than with the morphology of Asterids have been unanimous in their use of the term "cilia" (Gemmill, 1915; Irving, 1924, 1926; Budington, 1942). While one does not wish to argue about words *qua* words, it appears only proper to bring the description of these structures in Asterias into line with consistent modern terminology.[2]

The histology of the digestive tract in various Asterids has been the subject of several investigations varying greatly in thoroughness, accuracy, and interpretation of facts. Hamann (1885) describes in some detail the mucus gland cells of the pyloric caeca, referring to them as *Becherzellen*. He concludes that they form originally at the base of the epithelium as small cells which move toward the free border, increasing in size and finally establishing stomata at the lumen border. The

[2] In this connection, Hyman (1940, footnote, p. 375) comments upon the failure of workers on Coelenterates to distinguish between cilia and flagella and is herself careful to make this distinction in every justifiable case. It should also be mentioned that upon closer study of developmental stages of *Asterias forbesi* in living and freshly-fixed material, as well as in thin paraffin sections, it is apparent that the general "ciliation" of the blastula and gastrula, and the frequently cited "ciliated bands" of the larva, are actually formed by cells bearing each a single, long, vigorous flagellum. The illustrations, and in part the text, of Korschelt and Heider (1895) appear to bear out this interpretation. If, as seems likely, flagellated rather than ciliated epithelia are the rule among Echinodermata, this peculiarity is shared among very few of the animal phyla (Porifera, Coelenterata in part, scattered instances in vertebrate tissues) and makes a close comparison of echinoderm larvae with the annelid-mollusk trochophore even more difficult.

remainder of the cell population of the epithelium he does not analyze, grouping them in a brief discussion simply as "epithelial cells," each with one or more *Wimpern* (*"das erste scheint die Regel zu sein"*), and containing a finely-granular cytoplasm. Considering the staining methods used by Hamann (acetocarmine, Böhmer's hematoxylin) it is not surprising that he found no trace of the secretory cells with their abundant coarse granules.

Schneider (1902) was apparently the first to observe these secretory cells. He terms them *Eiweisszellen* and describes their coarsely-granular secretion-product as moving up to the free ends of the cells in two or three rows. His description of the mucus gland cells is somewhat more accurate than Hamann's, and he recognizes also a third cell-type, *Nährzellen*, otherwise undifferentiated. It is significant that Schneider does not ascribe a storage function to any of these types and considers the pyloric caeca as organs of secretion and possibly absorption.

MacBride (1906) writes simply that the cells forming the walls of the pyloric sac and its appendages are tall, narrow, cylindrical cells crowded with granules which appear to be of the nature of digestive ferments.

Chadwick (1923) first established the regional differentiation of cell-types characteristic of the Asterid pyloric caeca. "At two points which are practically in the median line of each caecum," he writes (p. 22), "the structure of the wall differs from that which forms the lateral sacculi. One of these is that portion of the wall of the caecum which lies between the points of attachment of the two mesenteries by which it is suspended from the aboral wall of the ray, the other is a deep fold which traverses its free oral face. In the latter the nuclei of the epithelial cells are much more densely crowded than are those of the sacculi, and in sections they appear as a fairly broad band. These cells bear especially long and powerful cilia." Here Chadwick is clearly referring to the cells which I have termed "special current-producers." Chadwick also describes and figures the vacuoles in the secretory cells, mistaking them for small mucus glands; he does not mention the secretory granules which always surround them.

The latest attempt to establish the histological details of the pyloric caeca of *Asterias* was that of Dorman (1928). Alone among those who have worked with this tissue, Dorman describes the epithelium as consisting of several layers of cells, to which by certain histochemical tests he ascribes different functions. According to this description, "hepatic cells" lie in two or three rows near the basement membrane and are rich in fat and glycogen; "pancreatic cells" occupy the inner part of the wall and are described as "exceedingly granular" and said to contain some glycogen. The remaining cells are "interstitial cells," present in large numbers, some squamous and some spindle-shaped.

Aside from the fact that such an attempt to perpetuate the "hepato-pancreas" concept of invertebrate midgut diverticula seems unnecessary, this description is widely at variance with the results of the present study. However, the sources of Dorman's confusion are evident. The architecture of this epithelium is such that a section passing through it at an angle to the long axes of the tall columnar cells could easily be interpreted as showing several layers of cells; in such a section no single cell would actually appear in its entirety. The illusion of layering is heightened by the variation in the location of nuclei in these cells. Moreover, the stratification of the carbohydrate and lipid reserves in these tall cells, so as to fill their basal ends while leaving the apices relatively empty, would in a poorly-oriented

section lead to exactly the conclusion drawn by Dorman. Unfortunately, the single figure of this tissue furnished by Dorman is of no assistance in substantiating this explanation.

Several investigators have reported results of experimentation and observation relating to the functions of the pyloric caeca in Asterids. The question of their role in digestion and absorption was long debated, Frenzel (1892) maintaining that their sole function was to secrete enzymes which passed into the stomach and acted there upon ingested food. Frenzel convinced himself that no food ever enters the caeca, and in order to account for the fat deposits observed in the cells of the caeca was forced to postulate that these cells absorb from the coelomic fluid fats previously digested in the stomach. Opposite conclusions were reached by Chapeaux (1893), who found no absorption in the stomach and demonstrated (a) that carmine grains fed along with fibrin were subsequently found in the caeca, and (b) that oil-fed animals later showed fat-droplets stored in the cells of the caeca. The presence of a variety of digestive enzymes in the caeca was unquestioned, having been detected in extracts both by Chapeaux and by Fredericq (1878).

Stone (1897) and van der Heyde (1923) were unable to demonstrate detectable amounts of glycogen in the walls of the caeca by chemical analysis. This fact, together with a series of experiments involving perfusion of the digestive tract with iron saccharate, ammonium carminate, and olive oil, all of which were later found in the caecal epithelium, led van der Heyde to the conclusion (p. 139) that "this liver, without doubt, like the livers of so many other invertebrates, is principally and primarily an organ of absorption."

That more is involved in the function of the caeca than simple absorption of foods previously digested in the stomach is clearly indicated by the experiments of Irving (1926). In this work Irving perfused surviving isolated caeca of the starfish *Patiria miniata* with solutions of gelatin in sea water and was able to demonstrate a progressive rise in the non-protein nitrogen levels in the sea water in which these caeca were immersed. This technique, well-controlled, yielded unquestionable evidence that the caeca can digest proteins, and that the products of digestion are passed through the epithelia into the surrounding medium. In the intact animal, this medium would be the fluid of the perivisceral coelom, which is chiefly responsible for the distribution of absorbed nutrients throughout the body.

The work of Dorman (1928), apparently misguided in its morphological aspects, still indicates that reserves are stored in the cells of the caecal epithelium. This conclusion is justified also on the basis of the present investigation. Glycogen, another polysaccharide complex, and lipids are all demonstrable in the storage cells of the caeca. The conclusion that these represent stored reserves rests upon the evidence, presented above, that they disappear upon starvation. Starvation for one or two weeks is sufficient to demonstrate a detectable decrease in these reserves, and prolonged starvation results practically in their disappearance. This disappearance is not, moreover, the result of general moribundity of the tissue; sections of the pyloric caeca after 8 weeks of starvation showed a completely normal epithelium with no apparent decrease in the secretory activity of mucus gland and zymogen cells.

In connection with the oil-perfusion experiments of Chapeaux and of van der Heyde, which (together with the absence of glycogen) largely motivated van der Heyde's assumption of absorption as the primary function of the caeca, one looks

in vain in their accounts for any indication that they examined for fatty deposits the caeca of animals which had not been fed on oil. As indicated by the results of the present study, such a simple control procedure would undoubtedly have shown that the caecal epithelium of any normal animal in a reasonably good nutritional state contains abundant stores of lipid. Lacking such controls, their arguments lose force.

It is clear, however, that absorption must occur before reserves can be stored; the abundance of alkaline phosphatase activity at the free borders of the storage cells furnishes another indication of the importance of the pyloric caeca as organs of absorption.

From the work of Cain (1947b) and others, one would expect to find deposits of histochemically-detectable phospholipids in connection with the Golgi element and mitochondria of these epithelial cells. As indicated above, however, the acid-hematein test, controlled by the pyridine-extraction test, has given consistently negative results with this tissue. It should be pointed out that the classical cytology of the epithelial cells of the pyloric caeca has apparently never been described, and the Golgi element and mitochondria of these cells have not as yet been recognized.

In summary, considering the evidence from all sources, the functional role of the pyloric caeca appears manifold. Particulate, partially-digested food is drawn from the stomach along Tiedemann's diverticula by the action of the strong flagellated cells localized here. Side currents (see Irving, 1924, and Budington, 1942) carry the particles into the lateral branches and maintain a circulation; a variety of enzymes from the secretory cells completes the digestion of the food (for a recent account of the proteolytic enzymes involved see Sawano, 1936); the products of digestion are absorbed by the storage cells, where some are elaborated into reserves of polysaccharide and lipoid nature while others pass directly through the wall of the caecum into the perivisceral coelomic fluid for distribution.

All studies concerned with the enzymatic complement of the caeca agree that proteins and carbohydrates are digested here, but a question remains as to whether fats are actually digested, or whether they are strongly emulsified and pass into the cells as minute droplets. Chapeaux (1893) held that no lipase was present and postulated that fat digestion was a function of the free amoebocytes of the coelomic fluid, which engulfed and digested fat droplets passed unchanged through the wall of the pyloric caeca. This interpretation of the role of amoebocytes was disproved by van der Heyde (1923), without, however, any evidence that lipolytic activity occurs in the caeca. The fat-storing proclivities of the storage cells indicate that they handle large amounts of lipid, and an investigation of the mechanism involved would be of considerable interest.

The significance of the apparently two-fold secretory products of the zymogen cells is also unknown. Many invertebrates secrete digestive enzymes in the form of fluids enclosed in vacuoles and do not form zymogen granules. It remains an interesting possibility that the secretory cells of the Asterid pyloric caeca, known to elaborate a variety of enzymes, secrete their various products in different forms.

## Summary and Conclusions

Histological and histochemical investigations of the pyloric caeca in *Asterias forbesi* reveal that their walls are composed of an outer splanchnic peritoneum made up of small, flagellated, cuboidal cells; layers of muscular, nervous, and connective

tissue fibers variously developed; and an inner epithelium generally composed of very tall, slender, flagellated cells bearing a dense brush border. Certain special areas (Tiedemann's diverticula) are lined with closely-packed cells termed "special current-producers," functioning to maintain directed movements of the contents of these central tubular cavities. Interspersed among these cells are numerous mucus gland cells. The walls of the lateral outpocketings of the central cavity are lined by an epithelium consisting of (a) secretory cells, producing conspicuous secretory (zymogen) granules and another product contained in small, clear vacuoles; and (b) storage cells, containing abundant lipids (almost entirely neutral fat), glycogen, and a polysaccharide-protein complex resistant to diastatic digestion. Mucus gland cells are less numerous in these areas. "Special current-producers" do not contain appreciable amounts of reserve substances, and the regions of the epithelium of which they form the lining do not show the vigorous alkaline phosphatase activity characteristic of the free border in the storage-cell areas. Starvation for 6 to 8 weeks results in a complete disappearance of all reserves but does not impair the secretory activities of mucus gland cells or of zymogen cells.

Previous works on the structure and functions of the Asterid pyloric caeca are summarized, and from all sources of evidence it is concluded that these organs function in digestion of food which passes into them from the stomach, in absorption of the products of digestion, and in storage of reserves. Transfer of nutrients to the coelomic fluid, for distribution throughout the body, is also a feature of their activities.

## LITERATURE CITED


BAKER, J. R., 1946. The histochemical recognition of lipine. *Quart. J. Micr. Sci.*, **87**: 441–447.

BUDINGTON, R. A., 1942. The ciliary transport system of *Asterias forbesi*. *Biol. Bull.*, **83**: 438–450.

CAIN, A. J., 1947a. Demonstration of lipine in the Golgi apparatus in gut cells of *Glossiphonia*. *Quart. J. Micr. Sci.*, **88**: 151–157.

CAIN, A. J., 1947b. The use of Nile blue in the examination of lipoids. *Quart. J. Micr. Sci.*, **88**: 383–392.

CHADWICK, H. C., 1923. *Asterias*. Liverpool Marine Biology Committee Memoir XXV. University Press, Liverpool.

CHAPEAUX, M., 1893. Sur la nutrition des Échinodermes. *Bull. Acad. Roy. de Belgique, Ser. 3*, **26**: 227–232.

DORMAN, H. P., 1928. The morphology and physiology of the invertebrate liver and hepatopancreas. *J. Morph.*, **45**: 537–554.

FREDERICQ, L., 1878. La digestion des matières albuminoïdes chez quelques invertébrés. *Arch. de Zool. Exper.*, **7**: 391–400.

FRENZEL, J., 1892. Beiträge zur vergleichenden Physiologie und Histologie der Verdauung. I. Mittheilung. Der Darmkanal der Echinodermen. *Müllers Arch. f. Anat. u. Physiol., 1892: 81–114.*

GEMMILL, J. F., 1915. On the ciliation of Asterids, and on the question of ciliary nutrition in certain species. *Proc. Zool. Soc. Lond.*, **1**: 1–19.

HAMANN, O., 1885. Beiträge zur Histologie der Echinodermen. Heft 2. Die Asteriden, anatomisch und histologisch untersucht. G. Fischer, Jena.

VAN DER HEYDE, H. C., 1922. On the physiology of digestion, respiration, and excretion in Echinoderms. Dissertation, Amsterdam.

VAN DER HEYDE, H. C., 1923. Petites contributions à la physiologie comparée. II. La résorption chez les Échinodermes. *Arch. Neerl. de Physiol.*, **8**: 118–147.

HYMAN, L. H., 1940. The invertebrates: Protozoa through ctenophora. McGraw-Hill Book Co., New York.

IRVING, L., 1924. Ciliary currents in starfish. *J. Exp. Zool.*, **41**: 115–124.
IRVING, L., 1926. Regulation of the hydrogen ion concentration and its relation to metabolism and respiration in the starfish. *J. Gen. Physiol.*, **10**: 345–358.
KORSCHELT, E., and K. HEIDER, 1895. Textbook of the embryology of invertebrates. Trans. by Mark & Woodworth. Macmillan, New York.
MACBRIDE, E. W., 1906. Echinodermata. Chap. 16 in Cambridge Natural History. Macmillan, New York.
SAWANO, E., 1936. Contributions to the knowledge on the digestive enzymes in marine invertebrates. 2. Proteolytic enzymes in the starfish, *Distolasterias nipon. Tokyo Bunrika Daigaku, Sec. B*, **2**: 26–43.
SCHEER, B. T., 1948. Comparative physiology. Wiley, New York.
SCHNEIDER, K. C., 1902. Lehrbuch der vergleichenden Histologie der Tiere. G. Fischer, Jena.
STONE, E. A., 1897. Some observations on the physiological function of the pyloric caeca of *Asterias vulgaris*. *Amer. Nat.*, **31**: 1036–1037.

# REDOX INDICATOR PATTERNS IN RELATION TO ECHINODERM EXOGASTRULATION.

## I. OXIDATION PATTERNS

C. M. CHILD

*Hopkins Marine Station and School of Biological Sciences, Stanford University, Stanford, California*

In a recent paper on gastrulation in the echinoid, *Dendraster excentricus*, following trypsin treatment, A. R. Moore (1952, p. 45) found that gastrulation occurred, even though the hyaline membrane had been removed by trypsin. Concerning this result he said, "This proves that the tensile force of the outer membrane is not an agent in gastrulation. The evidence leads to the conclusion that the process of infolding is the result of properties inherent in the vegetal plate" (Moore, 1952, p. 45). This conclusion, together with certain data concerning redox indicator patterns in echinoderm development, suggested to the present writer that further investigation of indicator patterns, particularly in relation to exogastrulation, might give some evidence concerning conditions which determine or are associated with entogastrulation and exogastrulation. The earliest observations on echinoderm indicator pattern concerned only patterns of intracellular reduction by decrease of oxygen content in the external medium after staining by oxidized "vital" dyes. As the title indicates, the present paper concerns only oxidative patterns: the data concerning reduction patterns are not complete, particularly as regards exogastrulation. It is hoped that, not only the earlier, but also more recent data concerning reduction patterns may be considered in a later paper. In the following data concerning oxidative patterns, particular attention is given to a feature of these patterns, not fully recognized in earlier work in consequence of the preoccupation with reduction patterns.

### Material and Methods

The echinoids, *Dendraster excentricus* and *Strongylocentrotus purpuratus*, and the asteroid starfish, *Patiria miniata*, have served as material, either at the Hopkins Marine Station, Pacific Grove, or at Palo Alto after transportation of the animals under slight refrigeration, and development in most cases at temperatures ranging from 18 to 20° C., or in some lots at approximately 13° C., or even 8–10° C. with *Dendraster*, during earlier stages. In *Dendraster* and *S. purpuratus* good fertilizations were obtained for three days after transportation to Palo Alto with *S. purpuratus* and for five days with *Dendraster* from animals kept in a refrigerator at 10–15° C., covered by seaweed or wet paper toweling and with a small amount of water in the container. Starfish material for the present purpose was fertilized

at Pacific Grove and brought to Palo Alto at once. *S. purpuratus* is more sensitive to environmental conditions than the other forms used.[1]

For intracellular oxidation a modification of the indophenol or Nadi reaction with very low concentrations of the two reagents, para-amino-dimethyl aniline (dimethyl-paraphenylene diamine) and α-naphthol as described repeatedly in detail in earlier papers (*e.g.*, Child, 1944, 1953b). Intracellular oxidation of the two reagents is catalyzed by an oxidase, regarded by some as cytochrome oxidase. With the low concentrations of the reagents and without the necessity of using alkali to dissolve the naphthol, the material is not killed at once and motile stages remain active until intracellular concentrations of indophenol become high. This modification of the Nadi reaction makes directly visible very slight regional differentials even more clearly in most cases than the catalyzed intracellular oxidations of dye solutions reduced by non-toxic concentrations of sodium hydrosulphite. This is largely due to the brilliant blue color of intracellular indophenol, even in very low concentrations.

In addition to the indophenol reaction various dyes, chiefly diazine green, methylene blue and in some cases Nile blue sulphate, reduced in external solution by sodium hydrosulphite ($NaHSO_2$ or $Na_2H_2S_2O_4$) were used. Minute amounts of hydrosulphite, fractions of a milligram, are sufficient to reduce one ml. of methylene blue 1/10,000 and the lower concentrations used of diazine green and Nile blue sulphate. This reducing agent has great advantages over the highly toxic reducing agents used in earlier work with redox indicators: those sometimes led to errors in results by retarding or completely inhibiting reduction in the most sensitive regions. Hydrosulphite is not appreciably toxic in concentrations and with exposure periods far above those required for dye reduction. Intracellular oxidation will occur in completely reduced external hydrosulphite dye solution if there is no great excess of hydrosulphite. The indophenol reaction and intracellular dye oxidation have been used repeatedly and results observed in many hundreds of individuals during the breeding seasons of most years from 1947 on.

Figures are essentially optical sections along the polar axis. Different magnifications are used in the three species. The egg and developmental stages of *S. purpuratus* are considerably smaller than those of the other forms and those of the starfish are larger than the *Dendraster* stages. Sizes of figures do not indicate the actual differences in size in the three forms but have been chosen chiefly in order to show the gradient patterns clearly. Figures of *S. purpuratus* are considerably more, those of the starfish less, magnified than the actual differences in size would require for representation. Intracellular indophenol reaction and dye oxidation are indicated by shading and by arrows pointing from regions of more rapid to those of less rapid reaction. Arrows external to the inhibited ectodermal regions of exogastrulae indicate only differentials along the polar axis without reference to differentials in the cell-wall. In the more extreme types of exogastrulae the ectoderm is more inhibited than entoderm and these differentials are slight and are indicated by short arrows, or they may be entirely absent. In all except one figure the entodermal differentials are indicated by shading.

[1] The kindness of the Director and staff of the Hopkins Marine Station in providing material and laboratory facilities, and in many cases for transporting the animals to Palo Alto, is gratefully acknowledged.

## Earlier Data Concerning Oxidation Patterns

The sand dollar, *Dendraster excentricus*, was the material for the earliest observations on oxidation patterns, and the indophenol reaction was used. Only the more important features of these patterns were discussed and these only briefly (Child, 1941b). In later cleavage stages and in blastulae of normal [2] development, the reaction was distinctly differential, decreasing in rate basipetally from the apical [3] region. In the gastrula and probably earlier, a ventrodorsal reaction gradient also became visible with ventral ectodermal region oxidizing more rapidly than dorsal. Within the blastocoel, mesenchyme apparently reacted more rapidly than entoderm, but with progress of invagination an entodermal gradient, decreasing basipetally from the tip of the archenteron, developed and rate of reaction at the tip increased still further with coelom formation. In still later stages the tips of the developing oral lobe and anal arms became the "high" ends of new ectodermal gradients. As the ciliated band developed, its cells also showed increase in reactivity above that of the general ectoderm. With gradual progress of starvation in the fully developed plutei the gradient differentials decreased, and before death almost completely disappeared. In these earlier data on oxidation gradient patterns it was not determined whether a differential in rate of oxidation was present between blastocoelar and outer surface of the cell-wall of the blastula and early gastrula, although it had been determined still earlier that dye reduction decreased in rate from the blastocoelar to the external surface (Child, 1936a, 1936b) and it had been suggested that this was probably due to lower oxygen content in the blastocoel than outside.

In a later, more extended study of indophenol reaction and reduction in normal *Patiria* development (Child, 1944) the rate of the polar indophenol oxidation gradient was found to decrease basipetally from the apical region in later oöcytes, cleavage stages, blastulae and early gastrulae. With progress of invagination a new oxidation gradient developed in the entoderm, as in *Dendraster*, with decrease in rate basipetally. A ventrodorsal oxidative gradient seemed to be visible in some late blastula stages, but is not indicated in the figures of these stages since it was difficult to determine when it first appeared. With further development it became more clearly visible and with coelom and stomodeum development other local oxidative patterns appeared. That paper, like the earlier studies of indicator patterns, in echinoderm development, was concerned primarily with polar pattern, and the indophenol reaction was allowed to continue until the polar and later local differentials became distinct, without much attention to the earlier stages of the reaction. However, it was indicated by the course of the arrows in certain figures, *e.g.*, Figures 11, 13–17, 19 and 20 (Child, 1944), that reduction in the cell-wall of blastulae and early gastrulae progressed from the blastocoelar surface outward. This differential was still regarded at that time as probably resulting from lower

[2] It is perhaps unnecessary to note that "normal" development indicates merely the range of variations occurring under those conditions which we regard as natural. In its origin and determining factors it does not differ in any way from the experimental modifications of development under other conditions.

[3] The terms "apical" and "basal" are used for earlier developmental stages as less awkward than "animal" and "vegetal" or "vegetative" and as permitting use of the terms "basipetal" and "acropetal" in description of gradient patterns.

oxygen content in the blastocoel than outside and therefore of minor importance in development. It was not even determined whether a differential in indophenol reaction, an oxidative differential, appeared in the cell-wall in early stages of the reaction. As will appear in the present paper, an oxidase differential is present in the cell-wall of the blastula and early gastrula. The failure to observe it in this earlier investigation of indicator pattern is an interesting example of the influence which a preconceived opinion may have on observation.

Redox indicator patterns of echinoderm exogastrulae have not been thoroughly studied. In the first attempt to learn something about indicator patterns of exogastrulae, only differential reduction of vital dyes was determined following staining by oxidized dyes with oxygen decrease in the external medium. These data are to be considered in a later paper. Patterns of intracellular oxidation of indicators have been determined only in *Dendraster* and only by means of the indophenol reaction and have been recorded only briefly (Child, 1941b). In that paper it was stated that in exogastrulae with enlarged and elongated entoderms indophenol reaction progressed from the entodermal tip toward the ectoderm. It was also noted that in the thick-walled blastulae which occur in exogastrulating agents and usually become exogastrulae, if not too much inhibited for further development, the cell-wall gradient progressed from the blastocoel outward, but the cell-wall oxidative gradient after actual evagination and elongation of the entoderm was not considered. In the less extreme types of exogastrulae in which the ectoderm approaches or attains pluteus form, the ectodermal oxidative pattern is essentially like that of the normal pluteus. In the more extreme forms of exogastrulae the ventrodorsal oxidative gradient is entirely absent. The polar gradient is present, at least in the evaginated entoderm, but may be completely absent in the ectoderm or that part of it which has not been entodermized. There again, it was not determined whether a cell-wall oxidative gradient was present in these elongated exogastrular entoderms.

In *Patiria* the indophenol reaction gradient, the oxidative gradient pattern, was determined only for normal development, and information concerning oxidative patterns of exogastrulae has been completely lacking up to the present.

## Oxidation Patterns of Normal Blastulae, Early Gastrulae and Exogastrulae of Dendraster

The polar pattern of the indophenol reaction and of oxidation of vital dyes reduced by sodium hydrosulphite in the normal blastula is a distinct gradient decreasing basipetally from the apical region (Fig. 1, *A*), like those of other echinoderms, so far as known (Child, 1941b, 1944, 1953b). However, the most interesting feature of the blastula pattern and one which has been largely neglected, as already noted, is the cell-wall gradient, decreasing from the blastocoelar to the external surface. It becomes visible with oxidation of vital dyes as well as with the indophenol reaction. Since this is an oxidative gradient pattern it evidently cannot be due to lower oxygen content in the blastocoel than outside. The immigrating mesenchyme cells react essentially like adjoining cells of the cell wall. The same polar and cell-wall pattern are present in the basal region and in the invaginating entoderm of the early gastrula (Fig. 1, *B*), but the ectoderm soon becomes so thin that presence of a cell-wall gradient in it becomes uncertain, *i.e.*,

the differential in the thin layer, if present, is not sufficient to be clearly visible, except in an apical thickening, as in Figure 1, *B*. In many individuals the cell-wall gradient still seems to be barely visible but it is not consistently distinguishable in all as development progresses. In general, the cell-wall gradient is most clearly visible in early stages of the indophenol reaction and dye oxidation. As intracellular concentration of indophenol or dye increases, it becomes progressively less distinct, and finally almost or quite indistinguishable.

Figure 1, *C* is a *Dendraster* exogastrula from a lot subjected to rather extreme change from low to high temperature. This and several other lots were kept after

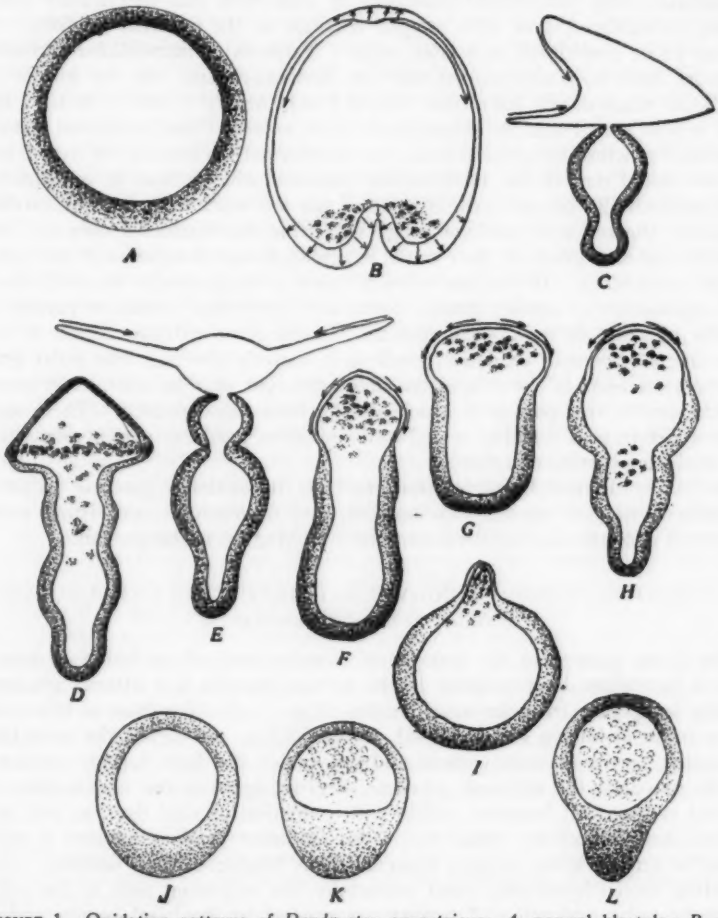



FIGURE 1. Oxidation patterns of *Dendraster excentricus: A*, normal blastula; *B*, normal early gastrula; *C-I*, LiCl exogastrulae; J-L, sodium azide pre-exogastrulae and early exogastrulae. For data concerning experimental conditions see the text.

fertilization at 8–10° C. and after 28 hours had developed to medium blastula stages. At this stage they were brought to a temperature of 20–22° C. for further development without any inhibiting agent. In the various lots this procedure resulted in exogastrulae ranging from an estimated 30 to 80–90 per cent, mostly of the less extreme types like Figure 1, *C*, with some degree of ventrodorsality and often approach to the pluteus form in the ectoderm. In the entoderm of Figure 1, *C* the polar gradient decreases from the tip toward the ectoderm and the cell-wall gradient from the external surface to the blastocoel, *i.e.*, opposite in direction to the cell-wall gradient in normal development.

Figures 1, *D–I* are various degrees of exogastrulation with exposure from the 4–8 cell stage to LiCl *M*/40 or *M*/50 for 24 hours or in Figure 1, *I*, 48 hours in *M*/50, followed by a day or two in water, at a temperature of 18–20° C. In *D*, as in *C*, the thin ectoderm is merely indicated in outline and gradient patterns by arrows. In the exogastrulae of *E-I* of Figure 1, the polar entodermal gradient decreases from the entodermal tip toward the ectoderm, and the cell-wall gradient from the external surface inward. This cell-wall pattern has been observed in many hundreds of *Dendraster* exogastrulae, the gradient of normal development, from the blastocoel outward, in none. In Figure 1, *E* the ectoderm is somewhat less inhibited than in *F-I* and its polar gradient is indicated by shading. In *I*, the ectoderm is almost absent and without distinguishable gradient.

Figures 1, *J*, *K*, and *L*, were in water for one or two days after 23 hours in sodium azide *M*/350 from the 8-cell stage at 20–22° C. throughout. In these, little or no differential recovery occurred after return to water. They are forms with thickened entodermal region, slight indication of evagination of entoderm and are pre-exogastrulae, or in *L* a slight degree of exogastrulation. In these, and large numbers of others in the same or lower azide concentrations, the polar entodermal gradient decreased from the tip toward the ectoderm, the cell-wall gradient from the external surface inward, as in LiCl exogastrulae, though usually with less differential than with LiCl. In these three examples of azide differential inhibition, the ectodermal cell-wall has remained relatively thick and the usual polar gradient was present in it, decreasing from the apical region, but with rather slight differential. A cell-wall gradient was also distinguishable in these ectoderms, decreasing from the blastocoel outward, *i.e.*, the same as in normal development. If reversal in direction of the entodermal cell-wall gradient is associated in any way with exogastrulation, reversal of the ectodermal cell-wall gradient is not to be expected when the course of its development is not altered. After a day or two in water entodermal dissociation, usually external, began in many individuals like Figures 1, *J–L* and the indophenol reaction was usually more rapid in the dissociated and apparently cytolyzed or cytolyzing cells than in intact entodermal cells.

The *Dendraster* blastula and early gastrula stages of Figures 1, *A* and *B* represent patterns observed in hundreds of control individuals. Their presence has been repeatedly confirmed by Dr. Olin Rulon. Also the entodermal patterns of Figures 1, *C–L* are selected cases only insofar as they are intended to show different degrees of exogastrulation and approaches to it. In all cases of the temperature, LiCl and azide forms, the entodermal cell-wall gradient is reversed in all degrees of evagination of entoderm and the approaches to it.

### Oxidative Patterns of Blastulae and Exogastrulae of Strongylocentrotus purpuratus

Developmental stages of *S. purpuratus* are smaller than those of *Dendraster* and *Patiria* and the cell-walls become so thin in the course of development that this form is somewhat less favorable material than the others, particularly as regards the cell-wall gradient. Moreover, it has seemed from earlier use of indicators that gradient pattern in this form has somewhat less differential than in the other two echinoderms. The polar gradient and the ventrodorsal gradient, when not obliterated by inhibiting conditions, are distinct in developmental stages, but after the cell-walls have become thin it is sometimes difficult to determine with certainty whether a cell-wall gradient is present. The inhibiting agent used for exogastrulation also decreases gradient differentials, but after return to water and more or less differential recovery, they may again increase to some extent. As starvation of the larvae progresses, the differentials of oxidative gradient pattern decrease and before death usually almost disappear. However, in normal development and in exogastrulae before the cell-walls have become very thin the differentials of gradient pattern are similar to those of *Dendraster*, though apparently less "steep."

In Figure 2, *A* the polar and cell-wall gradients of indophenol reaction in the normal blastula are indicated. The oxidation pattern of dyes reduced by hydrosulphite is similar. Earlier stages of *S. purpuratus* were also examined in an attempt to determine at what stage the cell-wall gradient became distinguishable. Often repeated examination of the earlier cleavage stages indicated that this gradient, decreasing from the blastocoelar surface, became distinguishable soon after a distinct blastocoel appeared, *i.e.*, about at 32- or 64-cell stages or perhaps somewhat earlier. At these stages the differential is slight and becomes distinguishable only in the earlier stages of intracellular oxidation. It has often seemed to be more distinct in the basal regions of the embryos, perhaps only because the cells of that region are larger than others. Certainly in later stages there is less differential basally than in the apical region (Fig. 2, *A*).

Also, often-repeated attempts have been made to determine whether the same cell-wall gradient is still present during invagination of the entoderm in normal gastrulation, as it is, at least in early stages, in *Dendraster* (Fig. 1, *B*). As invagination progresses, however, the cell-wall becomes so thin that, although in many cases the cell-wall gradient, decreasing from the blastocoel outward, seemed to be present in early stages of intracellular oxidation, its presence was still regarded as questionable.

In Figure 2, *B*, after 20 hours in LiCl *M*/20 at 18–20° C. without return to water, there is a slight basipetal polar gradient and in the apparently beginning evagination of the basal region, the prospective entoderm, indophenol and dye oxidations decrease from the entodermal tip, *i.e.*, from the outer surface inward, a reversal of th normal pattern. Figure 2, *C* is a somewhat similar case, also after 20 hours in LiCl *M*/20 at 18–20° C. Here a double gradient is present in the entodermal region, on the blastocoelar side a slight gradient decreasing from the blastocoel for a short distance, and externally another slight gradient, decreasing from the tip of the entodermal region and from the external surface inward. In this individual complete reversal of the entodermal cell-wall gradient has not yet occurred. The mesenchyme cells oxidize about as rapidly as, perhaps slightly more rapidly

than, the adjoining entoderm. The ectodermal polar gradient decreases basipetally and the cell-wall gradient remains as in normal development.

Figure 2, *D* represents an exogastrula two days in water after one day in LiCl *M*/40. In the thicker distal entoderm intracellular oxidation decreases from the entodermal tip and the cell-wall gradient from the outer surface inward. In the proximal entodermal segment a polar gradient is present with decrease toward the ectoderm, but the cell-wall has become so thin that presence of a cell-wall gradient is uncertain. In the ectoderm the polar and cell-wall gradients remain as in normal development.

The exogastrulae of Figures 2, *E* and 2, *F* were two days in water after 25 hours in LiCl *M*/50. Th entodermal polar gradient decreased from the tip, the cell-wall

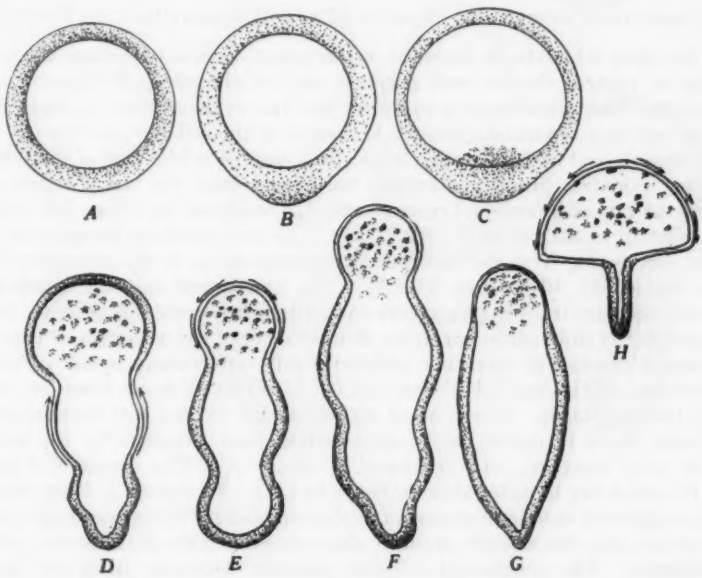



FIGURE 2. Oxidation patterns of *Strongylocentrotus purpuratus: A*, normal blastula; *B, C*, pre-exogastrulae; *D–H*, LiCl exogastrulae, some of mesenchyme cells becoming pigmented; data concerning experimental conditions in text.

gradient from the external surface inward. In the case of Figure 2, *E* the ectoderm retained a slight polar gradient but no cell-wall gradient was distinguishable; in the exogastrula of Figure 2, *F* no ectodermal gradient was visible.

The exogastrula of Figure 2, *G*, with the same treatment as *E* and *F*, was more inhibited than those, but with the same entodermal pattern and without distinguishable gradient pattern in the ectoderm. Figure 2, *H* from the same lot of eggs, with the same treatment and in the same container as *E–G*, is an example of the regional differences in inhibition which may occur in the same lot. The entoderm is more, the ectoderm less inhibited than in most animals of the lot, but the entodermal oxi-

dation pattern is the same as in *E–G*. The ectoderm is thin, and only a polar gradient is distinguishable.

Similar oxidative patterns have appeared in material two days in water following two days in sodium azide *M*/800 and *M*/1000 at the same temperature range as the preceding cases. Since these forms presented nothing new, additional figures are regarded as unnecessary. Thus far no exogastrulae of *S. purpuratus* without reversal of the cell-wall oxidative gradient have been seen, though in later stages of exogastrular life the entodermal cell-wall often becomes so thin that presence of a cell-wall gradient becomes questionable. Also in pre-exogastrulae and cases of slight evagination double entodermal cell-wall oxidative gradients are not infrequently present, as in Figure 2, *C*.

## Oxidative Patterns of Blastulae and Exogastrulae of Patiria

Of the three echinoderms included in this paper, *Patiria* is perhaps the most interesting as regards the cell-wall gradient and its alterations in relation to exogastrulation. The following data constitute the first evidence that an oxidation gradient, as well as a reduction gradient, is present in the cell-wall of *Patiria*. In the normal blastula and early gastrula the gradient pattern is like that of the echinoids. The polar oxidation gradient decreases basipetally from the apical region and at all levels the rate of indophenol reaction and dye oxidation decreases from the blastocoel outward in the cell-wall (Fig. 3, *A*). As the entoderm invaginates, a new gradient, decreasing from the entodermal tip, appears, as in the echinoids (Fig. 2, *B;* see also Child, 1944, Figs. 21–23). The entodermal cell-wall gradient persists, with decrease from the blastocoel outward; as the ectoderm becomes thin, the polar gradient is still visible, but in the thin cell-wall of the ectoderm of later stages it becomes impossible to determine whether a cell-wall gradient is still present.

The forms of Figures 3, *B–I* were in LiCl *M*/50 for 20 hours from late cleavage to early blastula stages. It was noted earlier (Child, 1953a) that these stages have been found more favorable for exogastrulation than exposure to the inhibiting agent in early cleavage. All developed at 18–20° C. The forms of Figures 3, *B* and *C* were a day in water after 20 hours in LiCl. In Figure 3, *B* the thickened, slightly evaginated entoderm shows an oxidation gradient decreasing from the outer basal surface and also a slight gradient decreasing from the blastocoelar surface of the entoderm. The ectodermal cell-wall gradient decreases from the blastocoel outward and the slight gradient on the blastocoelar side of the entoderm is merely persistence of a very slight differential of this gradient. In Figure 3, *C* the entodermal evagination has progressed much further and the cell-wall gradient decreases from the entodermal tip and from the external surface inward. In the thin ectoderm only a slight polar gradient is present as indicated. The exogastrulae of Figures 3, *D* and 3, *E* were two days in water after 20 hours in LiCl. In both, the characteristic entodermal exogastrular pattern and a slight polar ectodermal pattern appear. Figure 3, *E* is an example of the great entodermal elongation often appearing in exogastrulae of *Patiria*.

Exentogastrulae have been observed very generally in studies of echinoderm exogastrulation. They seem to occur more frequntly in *Patiria* than in the echinoids, but this may be merely a matter of degrees of differential inhibition and recovery or development of differential tolerance to the inhibiting agent. Figures 3, *F–I* are

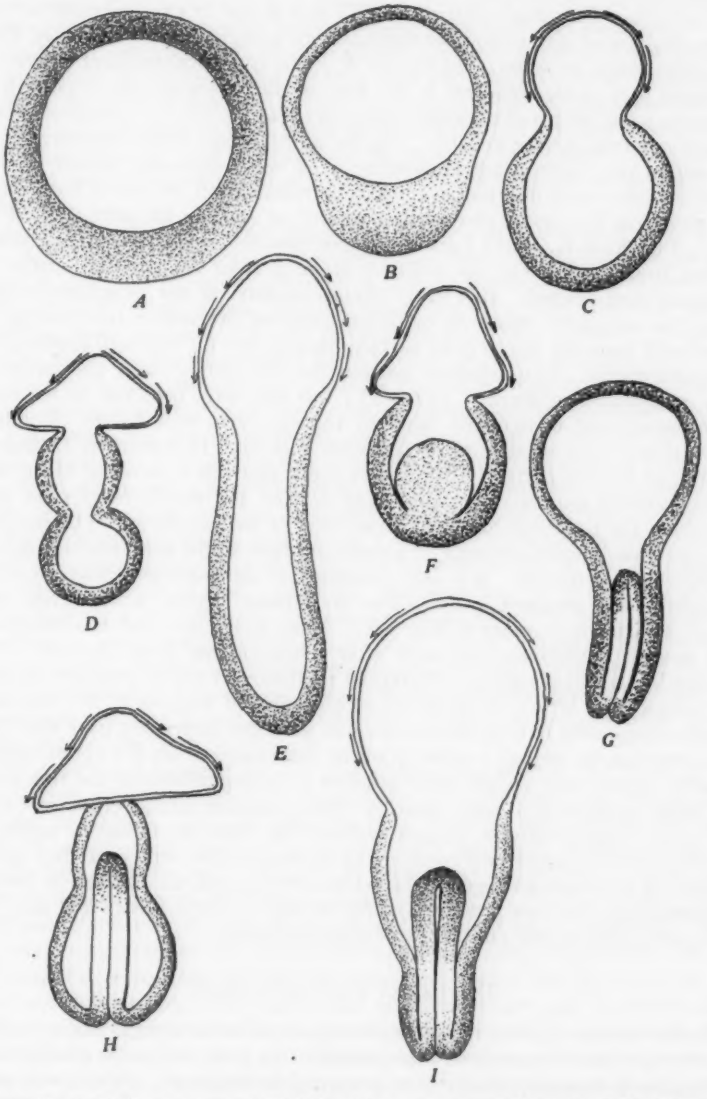



FIGURE 3. Oxidation patterns of *Patiria miniata: A,* normal blastula; *B-E,* different degrees of exogastrulation; *F-I,* exentogastrulae; all LiCl forms, further data in text.

examples of *Patiria* exentogastrulae in material 2–3 days in water after 20 hours in LiCl *M*/50. This concentration of LiCl and period of exposure did not prevent various degrees of differential recovery and an invagination of a part of the entoderm after return to water. In Figure 3, *F* the oxidation gradient pattern of the evaginated part of the entoderm is like that of other exogastrulae, a polar gradient decreasing from the tip of the evaginated region and a cell-wall gradient decreasing from the external surface inward. The invaginated part of the entoderm is a solid mass with a very slight gradient pattern. The most interesting feature of this pattern is that the slight differential present decreases from the tip of the invaginated mass and from its blastocoelar surface inward, at least in the part farthest invaginated. Figures 3, *G*, *H* and *I* are exentogastrulae with polar oxidation gradient decreasing from the tip, and the cell-wall gradient from the external surface of the evaginated part inward. In each of these exogastrulae the elongated invaginated part of the entoderm shows an oxidation gradient decreasing from its tip and in the cell-wall from the blastocoelar surface inward, like the cell-wall gradient of normal development. This gradient pattern did not extend over the entire length of the invaginated entoderm. In *G* and *I* this may have been due to the crowding of evaginated and invaginated parts of the entoderm and failure of indophenol agents to reach the more basal invaginated regions. However, in *H* there is no crowding of the two parts. It appears probable that this re-reversal of the cell-wall gradient pattern requires a certain length of time and that it takes place progressively from the tip along the invaginated part of the entoderm. The invagination and elongation indicate presence of a polar gradient in the invaginating region with its "high" end at the tip. It is also possible that the re-reversal may occur only at the higher polar gradient levels of the invaginated region; lower levels may not recover sufficiently to react in this way. Many years ago, and in numerous cases more recently, differential recovery after inhibition has been observed at higher gradient levels and persisting differential inhibition at lower gradient levels of the same echinoderm larva. In these exentogastrulae of Figures 3, *F–I* the ectoderm is thick enough only in *G* to show a cell-wall gradient decreasing from the blastocoel to the exterior, as well as a polar gradient, decreasing from the apical region. In the other figures only a slight polar gradient is distinguishable in the thin ectoderm.

*Patiria*, perhaps even more than the other available echinoderms, will undoubtedly repay further study of exogastrulation with different inhibiting agents. The data of the present paper on *Patiria* are by no means final, but, since they agree with the data on echinoids as regards the relation of the cell-wall oxidation gradient to exogastrulation, they are presented merely as part of the evidence that may perhaps throw some light on the physiology of exogastrulation.

## Discussion and Conclusions

Earlier studies of redox indicator patterns in echinoderm eggs and early developmental stages have demonstrated the presence of a polar oxidation gradient decreasing basipetally from the apical region in normal development. Also a new oxidation gradient appears in the invaginating entoderm, with decrease from the entodermal tip (Child, 1941a, 1941b; 1944, 1953b). This paper gives further evidence for the existence of these patterns and demonstrates their presence in exogastrulae, except when the ectodermal pattern is completely obliterated by the inhibiting agent.

In the earliest use of indicators on echinoderm developmental stages, only intracellular reduction of vital dyes with external oxygen decrease was considered. With this procedure a reduction gradient through the cell-wall of blastulae and early gastrulae was observed (Child, 1936a, 1936b). It was assumed, without further evidence, that this cell-wall gradient pattern, decreasing from the blastocoel outward, was merely an incidental result of lower oxygen content in the blastocoel than outside, in consequence of oxygen uptake by the cells of the cell-wall or the parts of these cells adjoining the blastocoel, at a higher rate than diffusion of oxygen inward from outside.

Presence of a cell-wall oxidation gradient was of course not recognized in these studies of differential reduction. However, it is now evident that an oxidation gradient, as well as a reduction gradient, is present in the cell-wall of the normal blastula and early gastrula and probably later, and that both gradients decrease from the blastocoel outward. In view of the presence of this oxidation gradient decreasing from the blastocoel outward, there appears to be no adequate basis for the earlier suggestion that the cell-wall reduction gradient, also decreasing from the blastocoel outward, results from lower oxygen content in the blastocoel than outside. That suggestion was never more than a hypothesis to account for the cell-wall reduction gradient. Actually nothing is known concerning oxygen content in the blastocoel, as compared with outside, but it appears improbable that the cell-wall oxidation gradient decreases from a region of lower, to one of higher, oxygen content. The cell-wall oxidation gradient is a feature of normal development; the reduction gradient appears only after oxygen decrease, either by oxygen uptake of the individual sealed in a small volume of liquid, or by use of a reducing agent. Perhaps under these conditions oxygen content in the blastocoel may become lower than outside in consequence of greater oxidase activity adjoining the blastocoel. The two cell-wall indicator gradients, both decreasing from the blastocoel outward, can be demonstrated in the same individual, though of course not at the same time. The oxidation gradient appears under natural conditions, the reduction gradient only after oxygen decrease.

In the evaginated entoderm of the exogastrula the cell-wall oxidation gradient decreases from the external surface inward, *i.e.*, it is reversed, as compared with the normal individual. This reversal has been observed only in that part of the exogastrula which evaginates as entoderm. This may vary widely in extent in either direction from the normal region of prospective entoderm. It may include only the most basal part of this region, as in Figure 2, *H*. In the earliest study of gradient pattern in exogastrulae it was also shown that in *S. purpuratus* and *S. franciscanus*, with delay of exposure to LiCl to late blastula stages, only the most basal part of the prospective entodermal region evaginated and developed as entoderm, or evagination might not occur, and entoderm might not be clearly distinguishable from ectoderm. At this stage of echinoid development entodermal activity is increasing and it is more inhibited than ectoderm. Incidentally, it is a question of some interest whether at this stage inhibited prospective entoderm is ectodermized. Certainly all degrees of apparent ectodermization appear under these conditions (Child, 1936b, Figs. 36–44). Similar restriction of evagination occurs in *Dendraster* under similar conditions (Child, 1940, Figs. 74–76). From forms of this character, often with even smaller evaginated entoderms with reversals of

oxidation gradients, at one extreme of exogastrulation, the region of evaginated entoderm and gradient reversal may extend in all degrees, not only over all of the prospective entodermal region, but also into the region of prospective ectoderm, until only a small knob of ectoderm remains in the apical region (Fig. 1, *I* above) or until the entire body is entodermized.[4] It is evident from the numerous studies on exogastrulation that, with exposure to the exogastrulating agent beginning in early developmental stages, the part of the individual undergoing evagination as entoderm in general extends farther apically as degree of effect of the agent increases. In a given lot of eggs, particularly if they are from different females, a wide range of sensitivity to exogastrulating action may occur. Consequently a wide range in the part of the animal undergoing evagination and reversal of the cell-wall oxidation gradient may occur in a single concentration of the agent. It is evident from these data that there is no regional difference in prospective ectoderm and prospective entoderm in early development which a single inhibiting agent cannot completely obliterate. It is also evident that in early stages lability increases basipetally, though perhaps not uniformly.

In normal pregastrular stages ectodermal development is more rapid and its gradient pattern soon becomes less labile than that of prospective entoderm, the apical region, the "high" end of the polar gradient, the least labile of all. It probably also becomes morphologically different from entoderm, as its further development suggests. With the gradual decrease in thickness of the ectoderm it becomes increasingly difficult to determine whether the cell-wall gradient persists in it. The basipetal differential in ectodermal lability apparently increases as the polar gradient differential increases from early stages onward.

It has seemed to be very generally true that the degree of inhibition necessary for entodermization of prospective ectoderm increases acropetally. It is necessary, however, to call attention here to certain forms of *S. purpuratus* appearing with inhibition by sodium azide, certain constituents of tobacco smoke and even with extreme crowding in water and in some cases with LiCl. In certain lots of material of this species all degrees of alteration of the apical ectodermal region from mere thickenings to outgrowths which are identical in appearance with evaginated entoderm at the basal pole of exogastrulae or in forms with invaginated basal entoderm appeared in large numbers in many containers. These apical outgrowths in many cases developed three segments, exactly like the basal entoderm (Child, 1948). In the first study of indicator patterns in exogastrulae somewhat smaller apical outgrowths, though often with three segments like basal entoderm, appeared very frequently with LiCl inhibition but were not included in published data, as it was desired to obtain further evidence as regards their occurrence. At present the only suggestion that seems to account for these forms is that the action of the agents was effective so early in development that alteration of the apical region occurred before its lability decreased appreciably. As the "high" end of the polar gradient it was more susceptible to inhibition and alteration than other levels of this gradient. If the agents were sufficiently effective very early in development, it seems possible that alteration and even entodermization of the apical region might occur without much effect on other levels of prospective ectoderm. *S. purpuratus* is in general more

[4] For earlier figures of this range of forms of exogastrulae see MacArthur, 1924, particularly Figure 2; Child, 1936b, Figures 1–5, 1940, various figures.

susceptible to inhibiting agents than *Dendraster* or *Patiria*. In *Dendraster* slight degrees of thickening and alteration of the apical region have been occasionally observed, but nothing that could be regarded as actual entodermization. Also only slight apical alterations have been observed in more recent *S. purpuratus* material. It is possible that the material of the 1948 paper was for some reason unusually susceptible or that the polar differential of early stages was greater than usual. Long experience with echinoderm material suggests that different lots may differ considerably in degree of reaction to external agents and in differential effects.

According to Figures 1, *J, K, L,* 2, *B, C, D,* and 3, *B* and *G,* above, there is no reversal in the cell-wall oxidation gradient as long as its ectodermal character persists. Apparently reversal occurs only when it is entodermized and takes part in evagination. The occurrence of reversal in the cell-wall oxidation gradient in evaginated entoderm raises what are perhaps the most interesting questions associated with exogastrulation. What determines reversal of this gradient? Does this reversal constitute a reversal of the physiological polarity of the entoderm cells or of the entodermal region in which it occurs? And finally, does the reversal determine reversal in the direction of entodermal growth, *i.e.*, evagination instead of invagination, or is it merely an incident or a result of an evagination determined in some unknown manner?

Considering first the question, how reversal occurs, it is evident that the cell-wall gradient represents a relatively slight differential. In a cell-wall only one cell thick it occurs between inner and outer ends of single cells. In solid entodermal masses it may involve multicellular regions. It is most clearly visible in the earlier stages of intracellular oxidation. As intracellular concentration of indophenol or oxidized dyes increases, it becomes difficult or impossible to distinguish it. In the presence of differentially inhibiting, exogastrulating agents this differential is undoubtedly decreased or perhaps completely obliterated with less inhibition than the polar pattern with much greater differential from apical to basal regions. The blastocoelar end of the cell-wall gradient, the region of higher oxidase activity, will undergo the greatest decrease. In various series it was observed in all three species that under inhibiting conditions the cell-wall gradient was very slight or could not be distinguished in many individuals which remained in blastula stages without definite invagination or evagination of entoderm.[5] As regards the intracellular conditions determining reversal of the cell-wall gradient in evaginating entoderm only suggestion is at present possible. Following decrease or obliteration of the original cell-wall gradient of evaginating entoderm, it appears highly probable that diffusion of oxygen inward, together with oxygen uptake of the cells, will establish a new gradient decreasing from the external surface. Less oxygen will reach those parts of the cells farthest from the external surface than those nearer the exterior.

[5] More attention was given to this point in *S. purpuratus* material than in the other forms. In laboratory records concerning this species decrease or absence of the entodermal cell-wall gradient was noted in blastulae 20 hours in LiCl *M*/20, two days in *M*/50, two days in *M*/60, in a lot with extreme crowding in water and in a lot one day in water after one day in sodium azide *M*/500. In *Dendraster* almost complete absence of this gradient was noted after 20 hours in azide *M*/600, after 20 hours in LiCl *M*/50, and in blastulae still living after a day in water following 20 hours in azide *M*/250. In various other lots presence of this gradient was uncertain but this was not specially recorded. These are believed to be cases in which the original cell-wall gradient was in process of being obliterated by the exogastrulating agent.

In various other organisms, particularly among the hydroids, an oxygen differential from the free surface, perhaps also an opposed carbon dioxide differential from the surface in contact with the substrate are apparently the factors determining a new polar gradient pattern and various other modifications of morphological pattern (Child, 1941a, pp. 413–420; also pp. 425–6 and Fig. 144). Moreover, oxygen may be an important factor in the experimental determination of ventrodorsality in *Dendraster* (Pease, 1941, 1942a, 1942b). Echinoderm material exposed to exogastrulating agents is usually returned to water before actual evagination occurs. Except in cases of extreme inhibition, this permits more or less differential recovery and still greater opportunity for determination of a new cell-wall gradient. It appears possible, however, that even without return to water, diffusion of oxygen into the cell-wall, together with the oxygen uptake of the cells, though oxygen uptake is less than under natural conditions, may determine a new gradient, decreasing from the external surface inward.

As regards the question of reversal of entodermal polarity by the reversal of the cell-wall gradient, it is to be noted first that physiological axiate patterns of morphogenesis and gradient pattern have been shown to be closely associated in many organisms, both in embryonic development and in reconstitution in later life. When ventrodorsal gradient pattern is obliterated by inhibiting agents in early echinoderm development, completely radial forms develop, like most of the exogastrulae in the figures of this paper. When gradient patterns are almost or entirely obliterated by an external agent morphogenesis is almost or completely absent (*e.g.*, Child, 1948, Figs. 79–85).[6] In the case of echinoderm exogastrulation there seems at present to be no reason for doubting that reversal of the cell-wall gradient reverses, partially or completely, entodermal polarity.

In view of the very general association of gradient pattern and course and character of development it appears highly probable that this reversal of the cell-wall gradient and entodermal evagination instead of invagination are directly associated. The entoderm invaginates when it possesses a certain cell-wall gradient. When this pattern is reversed it is highly probable that it must evaginate.

In the case of exogastrulation resulting from exposure to a very low temperature in earlier stages with transfer later to a room temperature of about 20° C. (Fig. 1, *C* above), the low temperature during the earlier stages evidently acts like other exogastrulating agents and decreases or obliterates the cell-wall gradient. On transfer to the much higher temperature, the relation between the greatly increased oxygen uptake and the diffusion of oxygen inward probably determines the reversal. The exogastrulae produced in this way in *Dendraster* are almost entirely of the less extreme types, with ectoderm approaching or attaining pluteus differentiation, *i.e.*, ectodermal recovery is almost complete, but reversal of the entodermal cell-wall gradient and evagination occur.

One other point remains to be considered. With certain degrees of differential inhibition exentogastrulae appear more or less frequently, usually with the less

[6] For another case of obliteration of gradient pattern and of morphogenesis see Child, 1941a, pp. 167–69, Figure 57 and pp. 425–6 and Figure 144. On the other hand, new patterns with a variety of symmetries in addition to the polar pattern develop in the reconstitution of isolated pieces of *Corymorpha* in relation to the differential originating between the surface in contact with the glass of the container and the parts freely exposed to the water (Child, 1941a; pp. 413–420, Figs. 141 and 142).

extreme degrees of inhibition and after return to water and differential recovery. They seem to occur more frequently in *Patiria* than in the echinoids and in many of these forms the entire length of the entoderm becomes very great, as in Figures 3, *G*, *H*, and *I*. Moreover, in these cases there seems to be a re-reversal of the cell-wall gradient in the terminal region of the invaginated part. Except for this terminal region the invaginated part may be crowded inside of the evaginated part and absence of any gradient pattern in it may be due merely to failure of the redox agents to reach it (Figs. 3, *G* and *I*), though in Figure 3, *H* the cell-wall gradient is evident only in the terminal region, and in that case there is no crowding. All of the exentogastrulae of these figures and various others were returned to water after 20 hours in LiCl *M*/50 at room temperature.

The change from evagination to invagination of the entoderm is the feature of greatest interest in these forms. As regards conditions determining this change, it is suggested that the reversal of the cell-wall gradient may not be quite complete in the evaginated entodermal tip, that some enzymatic or other trace of the original gradient may persist but may be obscured or overlaid by the gradient decreasing from the external surface. With differential recovery after return to water the tip of the evaginated entoderm recovers most rapidly and most completely, and the original cell-wall gradient may be to some degree reactivated and become sufficiently effective to bring about the beginning of invagination in the entodermal tip. If the beginning of invagination is determined in this way increase in the differential of this re-reversal of the cell-wall gradient may be expected to occur, at least in the invaginating tip. In the blastocoel the relation between diffusion of oxygen from the blastocoel into the cell-wall and oxygen uptake of the cells may be regarded as the factor concerned in this increase. In the terminal regions of the invaginated entoderms of Figures 3, *G*, *H*, and *I* and even in the invaginated entodermal mass of Figure 3, *F* this pattern is distinguishable.

With increase in concentration of the exogastrulating agent or increase in length of exposure period exentogastrulae decrease in frequency and with sufficient inhibition do not appear at all, even after return to water. This relation to concentration of agent and exposure period seems to indicate complete obliteration or destruction of any basis or substrate for the original cell-wall gradient. The reversed gradient pattern has apparently become irreversible in these cases.

Figures 3, *F–I* show only later or final stages of exentogastrulae. It has not been possible thus far to identify the beginnings of exentogastrulation, *i.e.*, the pattern of the entoderm at the critical point of the change from evagination to invagination. The indicators injure or finally kill and it is not certain that particular individuals which may suggest a change in gradient pattern would go on to definite exentogastrulation if they remained alive.

In the entexogastrula with invagination followed by evagination, the inhibiting conditions are at first not sufficient to determine evagination of entoderm but become effective later. Their occurrence may be determined by delay of exposure to the agent to slightly later developmental stages than those ordinarily used for exogastrulation or by increasing concentration of the agent after an initial exposure. In these there is merely the single change from invagination to evagination of entoderm.

Further studies of *Patiria* exogastrulation in 1953 indicate that at least some of those forms with both evaginated and invaginated entoderms are entexogastrulae,

rather than exentogastrulae. In those cases some degree of entodermal invagination is followed by evagination as effect of the inhibiting agent increases. Both invaginated and evaginated entoderm may continue to increase in length. In entexogastrulae the cell-wall gradient of the tip of the invaginated entoderm is not a "re-reversal" of pattern, but a persistence or a recovery of the original pattern, with decrease from the blastocoelar surface.

## Summary

1. A modification of the Nadi or indophenol reaction, using very low concentrations of agents, and intracellular oxidation of dyes reduced by sodium hydrosulphite, demonstrates the existence of a cell-wall oxidation gradient, decreasing from the blastocoel outward and from the apical region basipetally, in normal blastulae and early gastrulae of *Strongylocentrotus purpuratus, Dendraster excentricus* and *Patiria miniata.*

2. In the evaginated entoderm of exogastrulae, derived either from the original prospective entoderm or from entodermized ectoderm, the cell-wall gradient is reversed in direction, decreasing from the external surface toward the blastocoel.

3. The cell-wall oxidation gradient of ectoderm which persists as ectoderm does not undergo reversal, but as the cell-wall decreases in thickness, it becomes difficult or impossible to determine whether it persists.

4. Reversal of the cell-wall oxidation gradient by an exogastrulating agent is regarded as resulting from two factors: first, more or less complete obliteration of the original gradient by differential inhibition; second, from establishment of the reversed gradient by the relation between diffusion of oxygen inward from the external entodermal surface and the oxygen uptake of the entodermal cells.

5. It is suggested that this reversal of the oxidation gradient pattern reverses the polarity of the entoderm, and the reversed polarity determines evagination instead of invagination.

6. In exentogastrulae entodermal evagination is followed by invagination. In some of the exentogastrulae appearing in *Patiria* material a "re-reversal" of the cell-wall gradient occurs in the terminal region of the invaginated part of the entoderm. It is suggested that in the exentogastrulae some trace of the original cell-wall gradient persists, although the visible gradient is reversed. After differential recovery, following return to water, some degree of reactivation of the original gradient in the cell-wall of the entodermal tip, the region of most rapid and most complete recovery, determines the beginning of invagination, and conditions in the blastocoel bring about further reactivation in some individuals. Exentogastrulae decrease in frequency or do not appear at all with increasing degrees of inhibition, presumably because the original cell-wall gradient has been completely obliterated. In entexogastrulae there is no "re-reversal" of the cell-wall gradient but merely persistence or recovery of the original gradient.

## LITERATURE CITED

Child, C. M., 1936a. Differential reduction of vital dyes in the early development of echinoderms. *Arch. f. Entw.*, **135**: 426–456.

Child, C. M., 1936b. A contribution to the physiology of exogastrulation in echinoderms. *Arch. f. Entw.*, **135**: 457–493.

CHILD, C. M., 1940. Lithium and echinoderm exogastrulation. *Physiol. Zool.*, **13**: 4–42.

CHILD, C. M., 1941a. Patterns and problems of development. University of Chicago Press, Chicago, Illinois.

CHILD, C. M., 1941b. Formation and reduction of indophenol blue in development of an echinoderm. *Proc. Nat. Acad. Sci.*, **27**: 523–528.

CHILD, C. M., 1944. Developmental pattern in the starfish *Patiria miniata*, as indicated by indophenol. *Physiol. Zool.*, **17**: 129–151.

CHILD, C. M., 1948. Exogastrulation by sodium azide and other inhibiting conditions in *Strongylocentrotus purpuratus*. *J. Exp. Zool.*, **107**: 1–38.

CHILD, C. M., 1953a. Exogastrulation and differential cell dissociation by sodium azide in *Dendraster excentricus* and *Patiria miniata*. *Physiol Zool.*, **26**: 28–58.

CHILD, C. M., 1953b. Indicator gradient pattern in oöcytes and early developmental stages of echinoderms: a reexamination. *Biol. Bull.*, **104**: 12–27.

MACARTHUR, J. W., 1924. An experimental study and a physiological interpretation of exogastrulation and related modifications in echinoderm embryos. *Biol. Bull.*, **46**: 60–87.

MOORE, A. R., 1952. The process of gastrulation in trypsin embryos of *Dendraster excentricus*. *J. Exp. Zool.*, **119**: 37–46.

PEASE, D. C., 1941. Echinoderm bilateral determination in chemical concentration gradients. I. The effects of cyanide, ferricyanide, iodoacetate, picrate, dinitrophenol, urethane, iodine, malonate etc. *J. Exp. Zool.*, **86**: 381–404.

PEASE, D. C., 1942a. Echinoderm bilateral determination in chemical concentration gradients. II. The effects of azide, pilocarpine, pyocyanine, diamine, cysteine, glutathione and lithium. *J. Exp. Zool.*, **89**: 329–345.

PEASE, D. C., 1942b. Echinoderm bilateral determination in chemical concentration gradients. III. The effects of carbon monoxide and other gases. *J. Exp. Zool.*, **89**: 347–?86.

# REGENERATION OF THE ANTERIOR END OF AULOPHORUS FURCATUS (NAIDIDAE) WITH SPECIAL REFERENCE TO EFFECT OF X-RAYS [1, 2]

ROBERT HUGH FOULKES

*Department of Zoology, The State University of Iowa, Iowa City, Iowa*[3]

*Aulophorus furcatus* is an asexual, aquatic naid 3 to 7 mm. long and has the ability to regenerate very rapidly. The literature, however, reveals only taxonomic studies (Stephenson, 1930; Cernosvitov, 1944). Effects of irradiation on annelid regeneration have been almost entirely limited to total irradiation of varying doses (Stone, 1932, 1933; Turner, 1934, 1935). Zhinkin (1934) grafted lethally irradiated segments to normal hosts and attributed the resulting regeneration in the irradiated graft to a cell type, the neoblast, which had migrated from the healthy host. The neoblast is considered the vital factor by some investigators, while others believe it unimportant (Hämmerling, 1924).

In 1948, a technique of partially shielding an immobilized planarian exposed to x-radiation was reported (Wolff and Dubois, 1948). Their method was modified and applied to *A. furcatus*. Observations of the neoblasts in irradiated and non-irradiated worms were made in an effort to determine whether they contributed to regeneration in this species.

## Materials and Methods

Stock cultures of worms were maintained in glass dishes filled with distilled water and were fed boiled lettuce. Experimental worms were transferred to interlocking castor dishes. Amputations of the anterior ends were made at the sixth segment just posterior to the pharynx (Fig. 1). In the anteriorly irradiated worms, at least 8 and usually 12 to 15 segments were present between the wound and the shielded part of the worm.

The animals which had only the anterior half irradiated were immobilized by covering them with a thin layer of 2% agar. The agar containing the worms was cut into blocks. These were aligned on a watch glass and a three-mm. thick lead strip was placed over the posterior halves of the worms. They were then exposed to the determined lethal dosage of 2600 r at a rate of 300 r per minute. The radiation was produced by a 200 Kv air-cooled tube. A cardboard filter was used to minimize heat. Amputations of the head were made immediately after exposure.

[1] This investigation was carried out under direction of Dr. H. W. Beams, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology, The State University of Iowa, Iowa City, Iowa.

[2] Irradiation by permission and under direction of Dr. T. C. Evans, Radiation Research Laboratory, The State University of Iowa, Iowa City, Iowa.

[3] Present address: Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit 2, Michigan.

Three series of worms were observed in detail: one non-irradiated, one totally irradiated, and one of anteriorly irradiated worms. Totally irradiated animals died 14 to 21 days after exposure to 2600 r. Normal regeneration was completed during the fifth day. At selected intervals following amputation, the worms were fixed in Bouin's solution. Selected sagittal sections were stained with Delafield's hematoxylin and eosin Y and microphotographed at 600 diameters.

No grafting experiments were made. In preliminary work, it was observed that this species will discard any injured segment. Healing immediately follows and no fusion would occur. The fixative was also the killing agent as this worm rapidly autolyzes immediately following death. General observations were made on regeneration of the tail of normal and posteriorly irradiated animals.

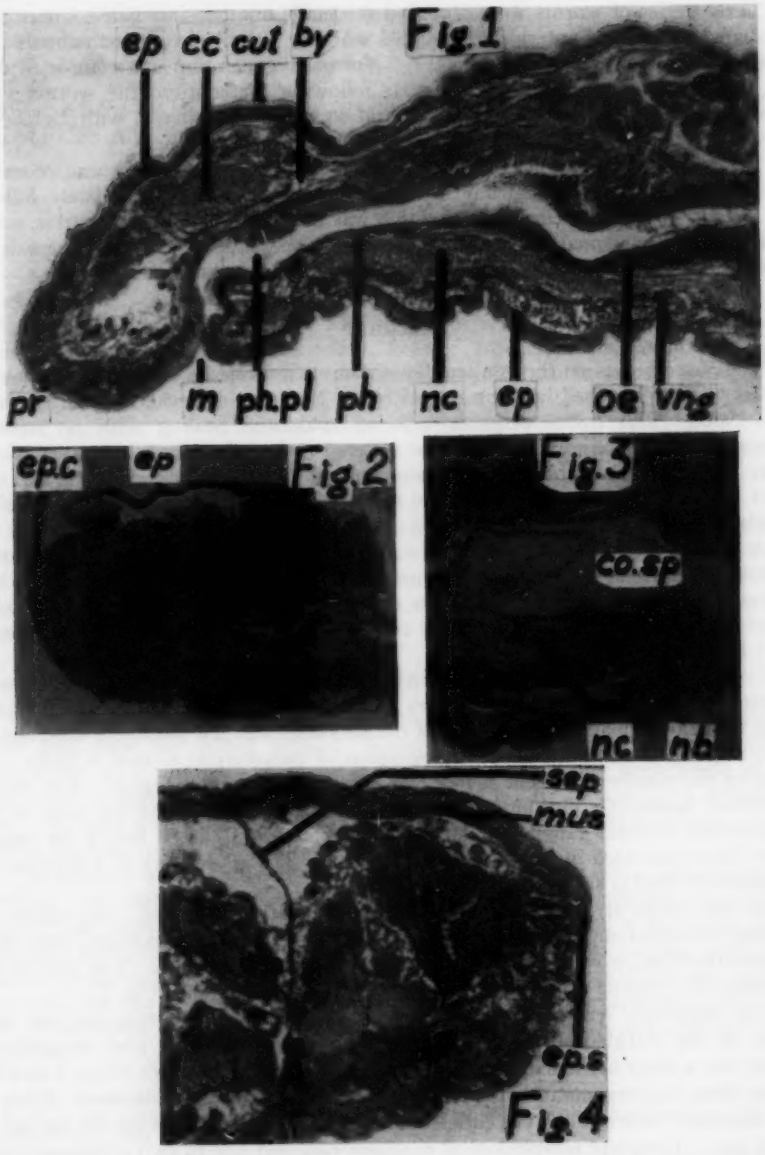
## Results

The observations on the non-irradiated worms indicated a sequence of recovery similar to that described in other species of the Naididae (Krecker, 1923; Stephenson, 1930). The initial response was an epidermal healing with no mitoses evident (Fig. 4). Simultaneously, the severed end of the alimentary tract closed over. In three to six hours, a migration of the neoblasts was evident (Fig. 3). Next, the epidermal scar thickened and some cell division occurred, after arrival of neoblasts. This thickening proceeded until the epidermal cap was formed, the newly formed cells seemingly pushed inward. These, together with the proliferating endodermal cells of the intestine, formed a column or cord of cells termed the cell strand. Between the dorsal side of the cell strand and the epidermal cap, a cluster of epidermal cells formed a primordial cerebral ganglion which was well developed after 12 hours.

Continued canalization of the cell strand formed the regenerated anterior alimentary tract. The muscular layers of the intestine developed into the thicker walls of the new pharynx. The fate of the neoblasts was not positively determined. That some neoblasts enter into the formation of mesodermal tissues is likely considering the numbers present before cell differentiations had begun. The neural structures apparently developed from existing ganglion cells and the cells of the regenerated cerebral ganglion. An average of five days passed before the regenerated head was used in feeding and locomotion. The appearance of a normal and regenerated head is shown in Figure 1.

In the totally irradiated specimens, no regeneration occurred. An epidermal healing succeeded amputation in two to four hours. The scar thickened more than in non-irradiated worms. Neoblastic activity was never evident. The size of the existing cells became progressively smaller until death resulted (Fig. 2).

The anteriorly irradiated worms had an immediate healing response and neoblasts in the shielded segments were active four to six hours after amputation. There was a delay in the initiation of regeneration of 12 to 24 hours (Figs. 5 and 6). Other than this, regeneration proceeded as in the non-irradiated worms. Some of the irradiated worms shed the cuticle from the exposed segments on the second or third day. Complete morphological recovery required one to two additional days. Functional recovery necessitated two to four days longer than in the non-irradiated worms.


ep
cc
cul
by
Fig. 1
pr
m
ph.pl
ph
nc
ep
oe
vng
ep.c
ep
Fig. 2
Fig. 3
co.sp
nc
nb
sep
mus
ep.s
Fig. 4

FIGURES 1–4.

In posterior regeneration, in both irradiated and non-irradiated worms, the neoblasts were seen migrating to the wound site. No detailed microscopy was made of posterior end regeneration.

## Discussion

The results indicated that the neoblasts, in this species, were involved in the regeneration process, either directly or indirectly. The presence of the neoblasts in the wound area contributed, in some way, to initiation of mitotic activity in the epidermal cap and cell strand. It is probable that the neoblasts form some of the regenerated mesodermal tissues. Several workers, using many neoblastic species, have concluded that the neoblast is essential to regeneration (von Wagner, 1906; Krecker, 1923; Sayles, 1927; Zhinkin, 1934). Contrary arguments as to the importance of neoblasts have been presented by other workers (Hepke, 1897; Abel, 1902). They attributed no function to the neoblast in the regeneration process. In all of these experiments, however, irradiation was not used.

Irradiation experimentation on two annelid species gives support to the contention that neoblasts, when present, do have an important role in regeneration. Irradiation of the neoblastic species, *Tubifex tubifex* (Stone, 1932, 1933) and *Lumbriculus inconstans* (Turner, 1934, 1935), revealed that the regenerative processes were halted or delayed. The conclusion was that the embryonic neoblasts were killed or severely inhibited. Similar experiments and results on species of planaria have been reported (Curtis, 1936). In *Rhynchelmis limosella*, irradiated segments were grafted to a non-irradiated host (Zhinkin, 1934). Regeneration in the graft began after the neoblasts had migrated from the host through the graft to the wound area. Observations on a planarian species in which one-half of the animal was irradiated have been reported (Wolff and Dubois, 1948). It was noted that regeneration in the distal portion of the irradiated half occurred after neoblasts had migrated from the non-irradiated portion to the wound area. No neoblasts were

---

Figures 1–4.

Abbreviations are as follows:

| | | | |
|---|---|---|---|
| bv. | blood vessel | m. | mouth |
| cc. | cerebral commissure | mus. | muscle layer of body wall |
| cg. | cerebral ganglion | nb. | neoblast |
| co. sp. | coelomic space | nc. | nerve cord |
| cs. | cell strand | ph. pl. | pharyngeal plate |
| cut. | cuticle | ph. | pharynx |
| ep. s. | epidermal scar | pr. | prostomium |
| ep. c. | epidermal cap | vng. | ventral nerve ganglion |
| ep. | epidermis | sep. | septum |

All sections are sagittal.
Magnification is 600 × except Figure 3 which is 900 ×.

Figure 1. Mid-sagittal section of head of normal worm.

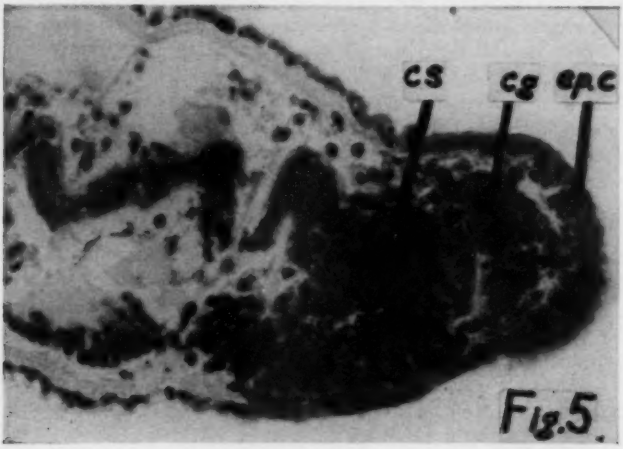
Figure 2. Anterior end of specimen 18 days following total irradiation of 2600 r. Head was amputated immediately following exposure. No cell strand. Reduced body diameter, all structures atrophied.

Figure 3. Two neoblasts migrating anteriorly along sheath of nerve cord. Large nuclei, prominent nucleolus. Section made of specimen six hours after amputation of head.

Figure 4. Four hours after amputation of head showing epidermal healing.

evident in the irradiated half. The most extreme interpretation of the regeneration role of the neoblasts was that of Hämmerling (1924). He compared the neoblasts to the meristem cells of plants or the interstitial cells of coelenterates.

The migratory ability of the neoblasts in *A. furcatus* was more extensive than that reported in other species. Migration toward the posterior end only was re-



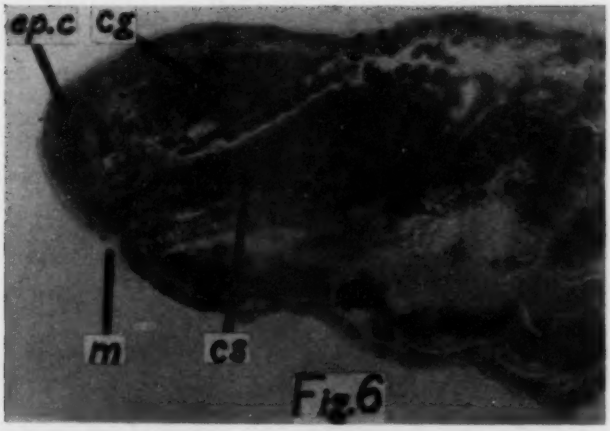




FIGURE 5. Forty-eight hours after head amputation of anteriorly irradiated worm. Many mitoses present. Mouth notch not yet present. Cell strand not as definite as in Figure 4.

FIGURE 6. Forty-eight hours after head amputation of normal worm. Cell strand well developed, mouth notch present. Cerebral ganglion well developed. Mitoses infrequent. Prostomium taking form.

ported in *Lumbriculus* (Sayles, 1927) and a maximum anterior migration of 7 to 9 segments in *Limnodrilus* and *Tubifex* (Krecker, 1923). In *A. furcatus,* anterior migration was observed through 15 or more segments.

The delay in the initiation of regeneration can be explained only in part by the time taken by the migration of the neoblasts. The general effects on other tissues (Clark, 1940; Lea, 1947) also contributed to the delay. Replacement of some cells and recovery from irradiation injury by the tissues, particularly the epidermis and endoderm, were necessary.

The posterior end of the worm is considerably different from that of the majority of oligochaetes. It has three pairs of gill-like processes and a pair of palps extending beyond the anal pore. Details of the histology of this posterior structure are yet to be recorded. Hence, posterior regeneration merited only general observation.

## Summary

1. A comparative study of regeneration of the head of *Aulophorus furcatus* in non-irradiated, anteriorly irradiated and totally irradiated worms has been presented. Totally irradiated worms failed to regenerate a new head. A delay in the initiation of regeneration and rate of regeneration was noted in the worms which had the anterior half exposed to lethal irradiation. The lethal irradiation apparently had its effect by killing or severely inhibiting the neoblasts.

2. In this species, it was demonstrated that the neoblasts are capable of migrating both anteriorly and posteriorly. They are able to migrate into irradiated areas. The neoblast apparently contributes, directly or indirectly, to the regeneration process of both non-irradiated and irradiated specimens. The neoblast probably forms some of the new mesodermal tissues.

## LITERATURE CITED

Abel, M., 1902. Beiträge sur Kenntnis der Regenerations-vorgänge bei den limicolen Oligochaten. *Zeit. f. Wiss. Zool.,* **73**: 1–74.

Cernosvitov, L., 1944. Oligochaeta from various parts of the world. *Proc. Zool. Soc. London, Ser. B,* **111**: 197.

Clark, G. L., 1940. Applied X-rays. Third edition, McGraw-Hill Book Co., New York and London.

Curtis, W. C., 1936. Effects of x-rays and radium upon regeneration. In: Duggar, B. M.: Biological Effects of Radiation. Vol. I, First edition, McGraw-Hill Book Co., New York and London, Chap. XII.

Hämmerling, J., 1924. Die ungeschlechtliche Fortpflanzung und Regeneration bei *Aelosoma hemprichii. Zool. Jahrb. abt. f. Allg. Zool. u. Physiol. der Tiere,* **41**: 581–586.

Hepke, P., 1897. Über histo- und organogenetische Vorgänge bei den Regenerationsprocessen der Naiden. *Zeit. f. Wiss. Zool.,* **63**: 263–291.

Krecker, F. H., 1923. Origin and activities of neoblasts in the regeneration of microdrilous Annelida. *J. Exp. Zool.,* **37**: 27–46.

Lea, D. E., 1947. Actions of radiations on living cells. Cambridge Univ. Press. Macmillan Co., New York.

Sayles, L. P., 1927. Origin of the mesoderm and behavior of the nucleolus in regeneration in *Lumbriculus. Biol. Bull.,* **52**: 278–312.

Stephenson, J., 1930. The Oligochaeta. Oxford Univ. Press, London.

Stephenson, J., 1931. The Oligochaeta: Reports of an expedition to Brazil and Paraguay in 1926–27. *J. Linn. Soc., London (Zool.),* **37**: 291–326.

STONE, R. G., 1932. The effects of x-rays on regeneration in *Tubifex tubifex*. *J. Morph.*, **53**: 389–432.

STONE, R. G., 1933. The effects of x-rays on anterior regeneration in *Tubifex tubifex*. *J. Morph.*, **54**: 303–320.

TURNER, C. D., 1934. Effects of x-rays on posterior regeneration in *Lumbriculus inconstans*. *J. Exp. Zool.*, **68**: 95–120.

TURNER, C. D., 1935. Effects of x-rays on anterior regeneration in *Lumbriculus inconstans*. *J. Exp. Zool.*, **71**: 53–82.

VON WAGNER, F., 1906. Beiträge zur Kenntnis der Reparationprozess bei *Lumbriculus variegatus* (*Gr.*), II Teil. *Zool. Jahr. Abt. f. Anat.*, **22**: 41–156.

WOLFF, E., AND F. DUBOIS, 1948. Sur la migration des cellules de regeneration chez les Planaires. *Rev. Suisse de Zool.*, **55**: supplement.

ZHINKIN, L., 1934. Über die Bedeutung der Mesodermanlagen bei der Regeneration von *Rhynchelmis limosella* (Untersuchungen über die Wirkung der Röntgenstrahlen auf Regeneration und Transplantation). *Zool. Anz.*, **105**: 305–312.

# A STUDY OF THE MECHANISM OF ACTIVATION AND NUCLEAR BREAKDOWN IN THE CHAETOPTERUS EGG[1]

LESTER GOLDSTEIN[2]

*Department of Zoology, University of Pennsylvania, Philadelphia 4, Pa., and the Marine Biological Laboratory, Woods Hole, Massachusetts*

The phenomenon of stimulation and response has been studied in a wide range of biological systems by a considerable number of investigators. Because of the many advantages of marine egg material, numerous workers have devoted their efforts to an examination of stimulation or activation[3] as it occurs in this material. These workers have believed that the phenomenon of stimulation and response is based on a fundamental cellular mechanism, the response to which, in eggs, is manifested by the initiation of some aspect of development. Some have limited their investigations to the maturation process, or more strictly the breakdown of the germinal vesicle nucleus, since they reasoned that this response was not complicated by as many factors as is cleavage.

The investigations reported here were concerned with nuclear breakdown in the immature eggs of the annelid *Chaetopterus pergamentaceus*. This material is especially favorable for study because of a number of characteristics not present in other available forms. For one thing, 100% of the eggs will show nuclear breakdown in a large majority of the worms, thereby insuring rather good reproducibility of results. Furthermore, nuclear breakdown occurs spontaneously in sea water, eliminating the necessity of artificial activation. These factors were in addition to the advantages existing also in other material—such as an easily detectable response and a short time period between stimulation and response.

Until 1950 only one paper, that of Allyn (1912), had appeared in which an attempt was made to explain, by experimental procedures, nuclear breakdown in Chaetopterus eggs. However, some work has been done on eggs of other forms. This earlier research on the process of nuclear breakdown includes the investigation of the effects of pH and ions on Pomatocerus eggs by Hörstadius (1923); a considerable amount of work, mostly concerned with effects of salts, by Dalcq (1928) and Pasteels (1935) on a variety of forms; experiments by Heilbrunn and Wilbur (1937) on the role of calcium ions in the initiation of nuclear breakdown in Nereis eggs; some work by Scheer and Scheer (1947) on the effects of

[1] This paper represents a part of a dissertation in Zoology presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Part of this investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by Dr. L. V. Heilbrunn.

[2] Predoctoral fellow of the National Cancer Institute, U. S. Public Health Service, 1952–53. Part of the work was performed during the tenure of this fellowship.

[3] In order to avoid any possible confusion with specific embryological terms, activation is defined here as any response to stimulation (either in egg cells or in other types of living cells). In this instance, it is the initiation of the cellular activity leading to nuclear breakdown.

drugs and ions in the activation of Urechis eggs; and some investigations by LeFevre (1945) concerning the effects of picric acid on the activation of Nereis eggs. There exists, of course, a much larger literature on fertilization and artificial parthenogenesis, phenomena which are related—in terms of activation—to the process of nuclear breakdown in oöcytes. The above mentioned papers, as well as reviews by Lillie (1941), Tyler (1941), Runnström (1949), Danielli (1951), and Heilbrunn (1952), may be consulted for a more extensive bibliography on the various aspects of activation in general.

So long as the eggs of Chaetopterus are retained within the parapodia of the worm their large (germinal vesicle) nuclei remain intact. This is apparent from the fact that if the eggs are examined within the first 5 minutes after they are shed, the large nucleus with its membrane can be seen in 100% of the eggs. Within 7 or 8 minutes after the eggs reach sea water, the nuclear membrane disintegrates and some of the nuclear material migrates to the periphery of the egg where the metaphase spindle of the first maturation division is formed. It has been the aim of this research (some of which is reported here) to answer, in physical and chemical terms, three questions relating to the phenomenon of nuclear breakdown as it occurs in the immature Chaetopterus egg.

Since the stimulus for the breakdown of the nucleus occurs as a result of the release of the egg from the animal into sea water, the first question posed was—is the initial activation a result of a direct external stimulus or is it a result of a release from an inhibition? As noted earlier in a preliminary note (Goldstein, 1950), the activation is apparently produced by the release of the egg from an inhibitory environment. This conclusion was based on the observations that nuclear breakdown would occur in the presence of any one of the four common cations (Na, K, Ca, and Mg), in the presence of any of several anions (Cl, $NO_3$, $HCO_3$, and Br), in sucrose solutions, in hypertonic and hypotonic sea water, at any pH between 3.5 and 9.0, and in the absence of oxygen. Thus, it was concluded that no factor in the sea water is responsible for the stimulus, but rather that activation results from a release from an inhibition. This view agrees with the opinion of Allyn (1912) and the results obtained by Scott and LeBaron (1950). The foregoing information led to the formulation of the second question, namely, what is the nature of the inhibitor present in the ovarian environment? Though a substantial amount of work has been done, most of the data concerning the inhibitor have been negative. At this time it is possible to say with assurance only that the inhibitor is heat labile and of a small enough molecular size to be dialyzable. (In a previous report (1950) it was stated that $CO_2$ might possibly be at least partially responsible for the inhibition. More recent and more extensive examination has indicated that this position is no longer tenable.) The third question is the one with which this paper is primarily concerned: What is the intracellular mechanism that produces the nuclear breakdown?

As a result of some early experiments, which revealed that calcium played an essential role in the activation process (*cf.* Goldstein, 1950), I was prompted to employ the colloid chemical theory of stimulation of Heilbrunn (1952) as a working basis for experimental design. This theory states, in part, that *any* stimulus applied to the cell will release calcium ions from a bound state, and that the calcium ions will then react with the protoplasmic colloids to produce the specific

cellular response. This view holds, moreover, that the mechanism is analogous to that involved in the clotting of blood. The approach employed here was that of "dissecting" the mechanism in such a manner that each individual step might be examined separately, following—to some extent—the approach used in interpreting the steps in blood clotting reactions.

I am grateful to Dr. L. V. Heilbrunn for suggesting this problem and for his willing encouragement, help and interest during the course of the work.

## Material and Methods

Male and female Chaetopterus worms, obtained from the Woods Hole, Mass., area, were kept in separate fingerbowls under running sea water. Worms kept in this fashion did not often shed gametes in the laboratory and could frequently be maintained in good condition for as long as two or three weeks. Eggs and sperm were obtained by cutting as many posterior parapodia from the worm as would supply sufficient material for a particular experiment, and then the parapodia were cut open (into the appropriate solution) to release the gametes. In those instances where the eggs were shed directly into solutions other than normal sea water, the worms were first rinsed with distilled water and dried externally with filter paper in order to prevent contamination with substances in the sea water. In order to be able to carry out certain experimental procedures within two minutes after the eggs were shed, some preliminary screening was carried out. As a result, a few deviations from the commonly accepted handling of Chaetopterus eggs were employed. Thus, it was determined that straining and washing of the eggs were ordinarily unnecessary for normal nuclear breakdown, once the parapodia were cut open in sea water. (Usually between 10 and 20 parapodia were cut into a dish containing 15–20 ml. of solution.) The processes under study were apparently unaffected by any extraneous material from the worm. Furthermore, it has also been observed that virtually no degree of crowding of the eggs would affect the progress of nuclear breakdown.

Two criteria for determining normality of the eggs were used. First, the eggs shed into sea water were expected to yield nuclear breakdown in 100 of 100 eggs. This expectation was realized in 90–98% of the worms. The second criterion, generally observed, was that nuclear breakdown would occur within 7 or 8 minutes after the eggs were placed in sea water. Counts were made of at least 100 eggs from a dish, except in experiments where it was necessary to make rapid counts, in which case only 50 eggs were counted. In all the work reported here the data were of such a nature as to warrant no further statistical treatment. To examine and count the eggs it was necessary to remove a drop of egg suspension to a slide and flatten the eggs with a coverslip. This was done in order to determine the presence or absence of the nucleus (but even without flattening it was often possible to see the clear polar region of the first maturation metaphase spindle).

In many experiments, though not in all, the normalcy of the eggs before and after treatment was determined by fertilizing the eggs upon their return to normal sea water. Fertilization (and subsequent cleavage) was taken as an indication of viability in a sufficient proportion of the experiments in a series to reveal whether or not a particular experimental technique involved irreversible damage. Experi-

ments with various types of inhibitors were considered valid only if the inhibition were reversible.

In utilizing $CO_2$ the following procedure was employed. The gas was bubbled into the solution through fine bolting cloth covering the opening of a glass tube leading from the tank. Since the gas was bubbled through under relatively low pressures, it was not possible (with the equipment available) to determine accurately the quantities of gas being delivered, and only rough estimates were made. Indeed, more exact estimates were hardly necessary and, in view of the fact that the material behaved in consistent fashion, the conclusions drawn from the experiments are believed to be valid.

Measurements of pH were all made with a Beckman pH meter.

In using the hand centrifuge for determinations of the rigidity of the cell cortex, the technique described by Wilson (1951) was employed.

Sea water modifications were made up according to Table I and were adapted, with certain alterations, from the analyses provided by Wattenberg (1938).

TABLE I

*Sea water modifications*

| | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | $MgSO_4$ | $NaHCO_3$ |
|---|---|---|---|---|---|---|
| Artificial sea water | 410 | 8.85 | 10.2 | 20.7 | 29.6 | 2.0 |
| Ca-free sea water | 428 | 9.3 | —— | 20.7 | 29.6 | 2.0 |
| Ca- and Mg-free sea water | 454 | 10.1 | —— | 5.7 ($K_2SO_4$) | 24.1 ($Na_2SO_4$) | 2.0 |
| Mg-free sea water | 426 | 9.7 | 10.2 | 5.7 ($K_2SO_4$) | 24.1 ($Na_2SO_4$) | 2.0 |

Figures represent ml. of molar stock solutions of the various salts. In each case, distilled water is added to make the final volume one liter.

The sodium citrate solution used throughout the investigation consisted of: 50 parts of 0.35 *M* sodium citrate plus 50 parts of Ca-free sea water. This solution will hereafter be called the *citrate solution*.

In analyzing for proteolytic activity, the method of Anson (1938) for trypsin determination with a hemoglobin substrate was followed as closely as possible. Incubation was carried out for one hour at 25° C. and the Folin-Ciocalteau reagent was added to color the reaction products.

## RESULTS AND DISCUSSION

### *Cortical changes*

Wilson (1951) has described physical changes in the protoplasmic cortex of Chaetopterus eggs during the mitotic cycle and these have been related to Heilbrunn's theory of stimulation and response. If the theory is correct, we should expect that somewhat similar changes would occur if the eggs were activated to produce nuclear breakdown. For reasons that will become evident shortly, a study was made of cortical changes in the maturing egg only during the first few minutes following activation (see Wilson for method).

Eggs were shed into artificial Ca-free sea water, which was somewhat inhibitory to nuclear breakdown.[4] *Two* minutes after the eggs were placed in the Ca-free sea water they were centrifuged at various rates for one minute, removed from the centrifuge tube, placed on a slide with a coverslip and examined for the presence of cortical granules. The absence of granules in the cortical region of the cell is taken as an indication that the cortex had become less rigid and could no longer resist the centrifugal forces applied.

Table II shows that the percentage of eggs in which the cortical granules were moved by centrifugation was always in agreement with the percentage of eggs showing nuclear breakdown as observed at 8 minutes after the eggs were shed. (The observations on the cortex were made at about 4 minutes after shedding—before the nuclei have broken down.) Further evidence was offered by the fact that if the eggs were centrifuged following nuclear breakdown, the eggs which retained intact nuclei always had a more rigid cortex. This alone, however, could not be considered seriously as proof of a correlation between cortical change and nuclear breakdown since it was impossible to control for the presence or absence of the nucleus and its effect on cortical rigidity. Therefore, the experiments described in Table II—in which all the eggs had intact nuclei at 2–4 minutes—were regarded as significant and indicated that those eggs in which the nucleus was destined to break down had a less rigid cortex.

TABLE II

*Nuclear breakdown and cortical rigidity*

| % eggs with cortical granules moved at 2–3 minutes | % eggs showing nuclear breakdown at 8 minutes | Centrifugal force in gravities |
|---|---|---|
| 28 | 25 | 4150 |
| 42 | 40 | 9340 |
| 10 | 9 | 2335 |
| 10 | 11 | 2335 |

A liquefaction of the protoplasmic cortex following the fertilization of Chaetopterus eggs has been reported by Wilson (1951). Moser (1939) has also described cortical changes following stimulation of Arbacia eggs. Wilson and Heilbrunn (1952) have interpreted these cortical changes to be a reflection of the release of calcium ions from binding to the cortical proteins and this, of course, would alter the consistency of the protoplasmic gel there. In this study it appears that the liquefaction of the cortex occurs as a necessary prelude to nuclear breakdown and the next sections will show what role calcium plays in the process.

### *Role of calcium*

That calcium plays a significant role in the activation of eggs has been shown by a considerable amount of work. Perhaps the most important was the contribution

[4] This solution was made up as follows: 94 gms. NaCl, 20 gms. $MgCl_2$, 15.7 gms. $Na_2SO_4$, 2.7 gms. KCl, 0.77 gm. $NaHCO_3$, 0.1 gm. $H_3BO_4$, and 3983 gms. $H_2O$. Eggs shed into this solution produced only about 10–40% nuclear breakdown. These intermediate numbers (rather than 0% or 100%) permitted a basis for comparing the frequency of cortical change with the frequency of nuclear breakdown. The inhibition was reversible, and so the solution proved to be a useful tool in this instance.

of Heilbrunn and Wilbur (1937). They demonstrated that the artificial induction of nuclear breakdown in Nereis eggs by ultraviolet light, NaCl or KCl was dependent on the presence of ionic calcium in the cell. If the eggs were placed in sodium citrate solutions, nuclear breakdown would not occur, citrate presumably making calcium unavailable for cellular activity. Repetition and extension of this work with Chaetopterus eggs was felt to be in order, since with this material no artificial measures are necessary for activation and, possibly, a different type of mechanism is at work.

Eggs were shed directly into a citrate solution (see Methods). This solution inhibited nuclear breakdown in ca. 90% of the eggs. If, 6 or 7 minutes after shedding into citrate, some of the eggs were placed in normal sea water and some were placed in Ca- and Mg-free sea water (see Table I), nuclear breakdown occurred in 100% of the eggs in normal sea water while a substantial percentage of those eggs in Ca- and Mg-free sea water was inhibited (see Table III).

These results suggested, of course, that divalent cations were necessary within the egg for activation to occur. When, however, the experiment was repeated, this

TABLE III

*Experiments indicating a requirement for divalent cations (Ca and Mg)*

| Eggs shed directly into normal sea water | Eggs shed directly into Ca- and Mg-free sea water | Eggs shed directly into citrate and then, at 6–7 minutes, transferred to: | |
|---|---|---|---|
| | | Normal s. w. | or Ca- and Mg-free s. w. |
| 100 | 100 | 100 | 40 |
| 100 | 96 | 100 | 18 |
| 100 | 99 | 100 | 7 |

(Figures indicate % eggs showing nuclear breakdown.)

time placing the eggs (from citrate) either into normal sea water or Ca-free sea water (Mg present), it was observed that again all the eggs in normal sea water produced nuclear breakdown, while there was a high percentage of inhibition of those eggs in Ca-free sea water as seen in Table IV. Apparently Mg is not the necessary cation since it is supplied in the Ca-free sea water. Therefore, it would appear that Ca is the important factor.

A third series of experiments (Table V), in which Mg-free sea water (containing Ca) was used instead of Ca-free sea water, proved to be conclusive. The results of these experiments showed no difference in the percentage of nuclear breakdown of the eggs when they were removed from citrate to either normal sea water or Mg-free sea water. Indeed, nuclear breakdown occurred in 100% of the citrated eggs in both Mg-free sea water and normal sea water. This experiment confirms the view that Ca and not Mg is important for the mechanism responsible for the dissolution of the nucleus.

The results of these three series of experiments (as well as a considerable amount of unpublished work) amply demonstrate the need for Ca in the mechanism of nuclear breakdown in the cell, and are in agreement with the work of Heilbrunn and Wilbur (1937). (The role of Ca in the activation of cells is discussed by Heibrunn,

TABLE IV

*Experiments indicating a requirement for calcium ions*

| Eggs shed directly into normal sea water | Eggs shed directly into Ca-free sea water | Eggs shed directly into citrate and then, at 6–7 minutes, transferred to: | | |
|---|---|---|---|---|
| | | Normal s. w. | or | Ca-free s. w. |
| 99 | 58* | 100 | | 6 |
| 100 | 52* | 100 | | 7 |
| 100 | 81* | 100 | | 24 |

(Figures indicate % eggs showing nuclear breakdown.)

* The controls in Ca-free sea water (without prior citrate treatment) were also inhibited (nuclear breakdown: 52–81%), but not to the extent that the eggs treated with citrate were (nuclear breakdown: 6–24%). It was shown (unpublished experiments done in another connection) by more conclusive means that, if 0.53 *M* NaCl were used instead of Ca-free sea water (right hand column of Table IV) calcium was again necessary for nuclear breakdown in eggs pretreated with citrate—whereas the controls in NaCl (without prior citrate treatment) showed 100% activation. Apparently the inhibitory effect of Ca-free sea water (without prior citrate treatment) here was due to some type of ion antagonism.

and his book (1952) should be consulted for a review of his theory.) Lindahl (1937) has questioned the results of Heilbrunn and Wilbur, claiming that the inhibition by citrate could very well be due to acidity. However, the work reported here shows that, following citrate treatment, the eggs may be placed in almost any environment lacking Ca and still be inhibited. It is only when Ca is returned to the cell that activation occurs and, consequently, the inhibition can hardly be attributable to a pH effect.

*Sequence of steps in the activation mechanism*

In view of the fact that $CO_2$ (Goldstein, 1950) and sodium citrate both reversibly inhibit nuclear breakdown, it was decided to investigate the possibility of a stepwise mechanism in which the inhibitors might be acting on different stages in the response mechanism.

A series of experiments was carried out in which the eggs were first placed directly from the ovary either into normal sea water saturated with $CO_2$ or into a

TABLE V

*Experiments indicating no requirement for magnesium ions*

| Eggs shed directly into normal sea water | Eggs shed directly into Mg-free sea water | Eggs shed directly into citrate and then, at 6–7 minutes, transferred to: | | |
|---|---|---|---|---|
| | | Normal s. w. | or | Mg-free s. w. |
| 100 | 91 | 100 | | 100 |
| 100 | 98 | 100 | | 100 |
| 100 | 100 | 100 | | 97 |

(Figures indicate % eggs showing nuclear breakdown.)

citrate solution, and then, after 8–10 minutes in one of these solutions, were removed to the other inhibiting solution. Examination showed that nuclear breakdown would be prevented no matter in what sequence the inhibitors were applied. These facts in themselves give virtually no insight into the mechanism of activation, though the information obtained was useful for later interpretations.

A further series of seven experiments proved to be more revealing. If the eggs were shed initially either into 0.53 *M* NaCl (non-inhibitory) or into normal sea water, allowed to remain for three minutes (at 21° C.) and then removed either to normal sea water saturated with $CO_2$ or to a citrate solution, nuclear breakdown was inhibited only in those eggs in $CO_2$-saturated sea water.

The results of these experiments suggested a tentative hypothesis which was substantially confirmed and elaborated by later experiments. The data suggest that calcium must perform its (yet to be described) function during the first three minutes after the initial stimulus and that after this function is fulfilled, calcium is no longer needed—since citrate can no longer inhibit. Allen (personal communication) has also shown, in Spisula eggs, that the activation process is sensitive to the lack of calcium during the first four or five minutes after the stimulus is applied. The inhibition by $CO_2$, both by immediate immersion and immersion after three minutes, presented a more puzzling problem—though not an insoluble one. The immediate inhibition can be explained in at least two ways. $CO_2$ could either inhibit the release of calcium from binding (possibly in the cortex) or it might prevent calcium from performing any function once it had been released. (It is doubtful that $CO_2$ inhibits calcium release. In fact, later data indicate just the opposite—that $CO_2$ produces a release of calcium from binding with protein, for it is expected that in the presence of a sufficient concentration of $CO_2$ the protein involved is on the acid side of its isoelectric point and is probably no longer capable of binding cations.) Furthermore, $CO_2$ would appear to inhibit some step in the process that follows both calcium release and the immediate effect that such released calcium produces. This is evident from the fact that calcium apparently completes its function during the first three minutes following the initial stimulus and yet $CO_2$ is still capable of inhibiting after this time. (These relationships will be more clearly demonstrated by the evidence presented in the next section.) The release of calcium that has been mentioned has not been demonstrated directly for the Chaetopterus egg, though it has been shown for other forms (Mazia, 1937 and Örström and Örström, 1942). The evidence for the existence of this phenomenon in Chaetopterus eggs has been shown indirectly by Wilson and Heilbrunn (1952) and is suggested here by the time relationships of cortical liquefaction (at about two minutes after the eggs have been shed) and the functioning of calcium (at about three minutes after the eggs have been shed).

After the above experiments were performed, a serious criticism came to mind. If there were a significant difference in the rate of penetration of citrate and $CO_2$ into the cells, many of the conclusions based on the data (regarding time of action) from this entire section would not be valid. For example, the fact that $CO_2$ could inhibit nuclear breakdown following three minutes immersion in sea water, while citrate could not, might be a result of the ability of $CO_2$ to penetrate more rapidly and thus affect a reaction that was still sensitive; whereas if the citrate were slower in entering the cell, the reaction might be complete before citrate could be effective.

Fortunately, however, later experiments (see next section) indicate that the general conclusions are substantially correct.

*Evidence for the existence of a calcium-activated component*

In the previous section, the suggestion was made that after calcium is released from the cortex and during the first three minutes after the eggs leave the ovary, some factor or system—not well defined—was acted upon (or activated) by calcium. This view was based on some indirect evidence. However, as was intimated, some much more conclusive data are available to establish the fact that calcium activates another component.

The experiments to be described were made feasible by an earlier observation that if the eggs were shed into a solution of 0.53 *M* NaCl saturated with $CO_2$, not only would nuclear breakdown be inhibited but if the eggs were left in the solution long enough, the calcium of the eggs would leak out. Presumably $CO_2$ caused calcium to be released from binding with protein, and calcium ions were then able to diffuse out of the cell to a region of lower concentration, there being none in the external medium.

(Examination of Figure 1 will be helpful in understanding the description of the experiments to follow.) Two parallel series of experiments were run. In the first, eggs were shed directly into $CO_2$-saturated 0.53 *M* NaCl and allowed to remain in that solution for 12 minutes, during which time nuclear breakdown was inhibited and presumably the calcium of the eggs diffused out into the environment. After 12 minutes some of the eggs were transferred to 0.53 *M* NaCl (dish A in Fig. 1) and some other eggs were transferred to a solution made up of 7 parts of 0.53 *M* NaCl and 3 parts of 0.30 *M* $CaCl_2$ (dish B). If the idea presented above is correct, then nuclear breakdown could not occur in dish A—calcium having been lost before activation was effected. On the other hand, nuclear breakdown would be expected to occur in dish B since calcium was returned to the cell and again made available for activation. In the parallel experiment, eggs were shed directly into 0.53 *M* NaCl and allowed to remain in that solution for three minutes, during which time calcium would be released and functioning. After three minutes the eggs were placed in $CO_2$-saturated 0.53 *M* NaCl, as in the above experiment, and nuclear breakdown was inhibited during the time that the eggs remained in that solution. The eggs were left in this latter solution for 12 minutes, during which time, again, the calcium should have leaked out of the cells. At the end of the 12-minute period some of the eggs were placed in 0.53 *M* NaCl (dish C) and other eggs were placed in a solution composed of 7 parts of 0.53 *M* NaCl and 3 parts of 0.30 *M* $CaCl_2$ (dish D). According to the hypothesis, nuclear breakdown is expected to occur in both latter instances with calcium present or not, since calcium activation should have occurred during the first three minutes in 0.53 *M* NaCl. Therefore, it is expected that nuclear breakdown would result in both dishes C and D. The predictions for dishes A, B, C, and D were realized. In a typical experiment (in all, four experiments were performed) the following results were obtained:

| Dish | % nuclear breakdown |
|---|---|
| A | 22 |
| B | 98 |
| C | 93 |
| D | 88 |

The critical feature to note is the difference between A and C, though both were devoid of calcium. This evidence is considered as demonstrating that calcium participates in the activation of some component during the first three minutes following the release of the egg from the ovary, and that this calcium-activated factor no longer requires the presence of calcium as it acts more directly on the nucleus. (The process is in this way analogous to the activation of prothrombin to thrombin in blood clotting—a reaction which is also dependent on the presence of calcium. Also, as in the cellular process above, following the activation of thrombin, calcium

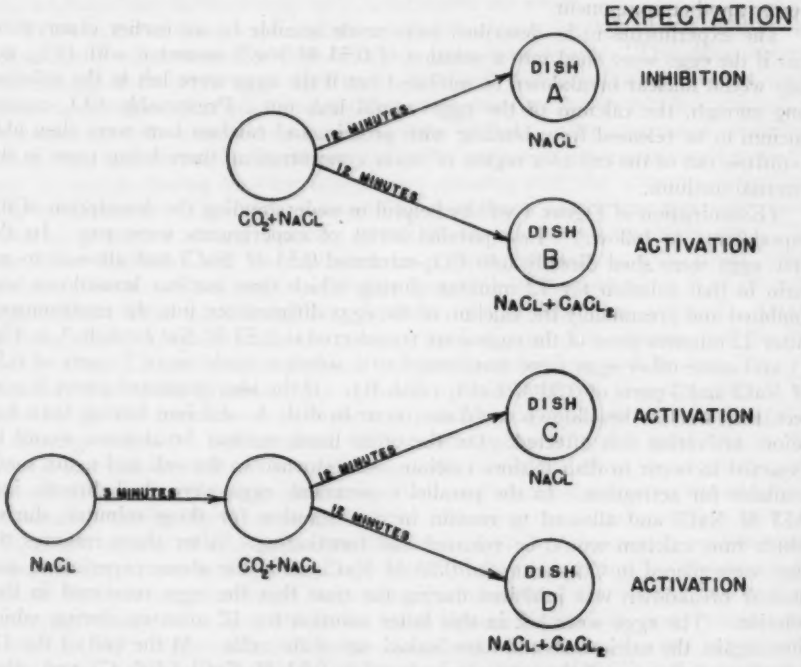



FIGURE 1. Experiment showing the existence of a calcium-activated component. See text for experimental details.

may be removed from the medium without inhibiting the subsequent reactions in the clotting process.)

It can be seen that calcium functions at approximately the same time, or shortly after, the cortex loses its rigidity. This evidence suggests that the two phenomena are closely associated and that, presumably, calcium is released from binding in the protoplasmic cortex—resulting in the loss of rigidity. Admittedly, it is impossible to state unequivocally that the *released* calcium is responsible for the activation resulting in the breakdown of the nucleus, since it is possible that the amount of free calcium ions present before stimulation was adequate for nuclear breakdown to have

occurred. However, there is probably very little free calcium present in the unactivated egg protoplasm. This is certainly true for the sea urchin egg (Mazia, 1937).

*Nature of the calcium-activated component*

Since the calcium-activated factor may be acting directly upon the nuclear membrane, which is presumably protein or lipoprotein in character, at least three possibilities as to the nature of the factor were envisioned. (1) Conceivably the activated factor could be acting as a reducing agent to convert S-S linkages to -SH radicals in the protein, thereby leading to a dissolution of the architecture of the membrane. Experiments utilizing -SH and S-S compounds, as well as reagents affecting these groups, established that this probably could not be the mechanism operating. Several reagents were used but most of the experiments were performed with glutathione, cystine and p-chloromercuribenzoic acid, none of which behaved as would be expected from the above hypothesis. (2) It is possible, assuming the presence of lipid in the membrane, that a lipase digests part of the structure and thus breakdown of the nucleus results. This possibility has not been tested. (3) The most appealing hypothesis appears to be that the calcium-activated factor is a proteolytic enzyme.

The foundations for the third view are as follows:

(a) Calcium is known to activate, or participate in the activation of, some proteases. (However, this also applies to some other enzymes, including lipases.)

(b) The nuclear membrane is probably mostly protein and appears to be dissolved during maturation.

(c) Yamane (1930) has reported some experiments which suggest a protease participation in some phase of the maturation mitosis of mammalian ova. Bohus Jensen (1948) and others have also described a certain degree of "nuclear activation" of sea urchin eggs by commercial trypsin.

a. Behavior of trypsin

In order to ascertain if a proteolytic enzyme is capable of producing nuclear breakdown, experiments were carried out utilizing crystalline trypsin (obtained from Worthington Biochemical Laboratory).

(1) Eggs were shed directly into a citrate solution in order to remove the calcium from the cells. After 8 minutes in that solution, some of the eggs were placed in a 0.02% trypsin solution made up in Ca-free sea water, and some eggs were placed in Ca-free sea water without trypsin. The first four items in Table VI show the results of this type of experiment. In every case, though nuclear breakdown took as long as 20–25 minutes for some eggs as compared to the normal time of 7–8 minutes, the eggs which were placed in the trypsin solution gave a substantially higher percentage of nuclear breakdown—though no significant amount of calcium was present in the eggs.

(2) As a check against any possible contamination of the trypsin preparation, another set of experiments was performed in which a trypsin inhibitor was used. In these experiments, following the immersion in citrate for 8 minutes, some of the eggs were transferred to Ca-free sea water, some of the eggs were transferred to 0.03% trypsin in Ca-free sea water, and some were placed in 0.03% trypsin in Ca-free sea water to which was added crystalline soybean trypsin inhibitor (obtained

from Worthington Biochemical Laboratory) to make a 0.03% concentration of that substance. The results of this experiment are shown in the last four items in Table VI. It can be seen, from the figures in the right hand column, that the inhibitor completely blocked the action of trypsin in producing nuclear breakdown. Apparently, then, it was the action of the enzyme itself which was responsible for the breakdown of the nucleus.

(3) Since trypsin is a fairly large molecule, there was some doubt as to whether the enzyme was actually entering the cell. Possibly trypsin was simply acting on the surface to activate, or release, some factor which would act more directly on the nuclear membrane. To determine if trypsin actually penetrates into the cell, trypsin solutions were made up in acid Ca-free sea water (pH 3.5–4.0) and then tested. Following the same procedures as in (1) above, three experiments showed that eggs placed in trypsin in acid media produced substantially as high a percentage

TABLE VI

*Effect of trypsin on nuclear breakdown*

| Exper. No. | Concentration of trypsin | % Eggs showing nuclear breakdown in: | | |
|---|---|---|---|---|
| | | Ca-free sea water | Trypsin | Trypsin and inhibitor |
| 1 | 0.02% | 31 | 49 | |
| 2 | 0.02% | 5 | 34 | |
| 3 | 0.02% | 5 | 57 | |
| 4 | 0.02% | 4 | 37 | |
| 5 | 0.03% | 10 | 65 | 8 |
| 6 | 0.03% | 8 | 81 | 10 |
| 7 | 0.03% | 2 | 26 | 3 |
| 8 | 0.03% | 9 | 34 | 10 |

of nuclear breakdown as those eggs placed in trypsin in ordinary Ca-free sea water (pH 8.0–8.2). Since, under these circumstances, trypsin is effective at an external pH at which it can not perform proteolysis, the data suggest that the enzyme is entering the cells where the cytoplasmic pH is relatively unaffected by the external hydrogen ion concentration.

Thus we see that trypsin can act within the cell to produce nuclear breakdown in the *absence* of calcium. Consequently, the enzyme appears to be performing the same function as the aforementioned calcium-activated component. Encouragement for these views is offered by the recent observation of Callan (1952) that trypsin is capable of dissolving the membrane of isolated nuclei of Triturus oöcytes.

b. Protease of the eggs

Encouraged by the results of the trypsin experiments, attempts were made to isolate and characterize the intracellular protease—if present. The results to date are preliminary and should only be considered as suggestive

All the eggs from approximately 5 worms were shed into about 35 ml. of Ca-free sea water—the process required about four minutes. After mixing to insure equal distribution, the suspension was divided into two parts of 15 ml. each. One

portion was frozen immediately in a bath of solid $CO_2$ and methyl Cellosolve (at ca. − 75° C.). The eggs in the other fraction were centrifuged and re-suspended in 15 ml. of normal sea water and allowed to stand for another 8 minutes, after which time (about 14 minutes after the initial shedding) that suspension was also frozen. The two preparations were then dehydrated by freeze-drying. The dried preparations were re-suspended in 5 ml. of distilled water each and allowed to stand for two hours in a refrigerator at 6° C. After two hours in the refrigerator, the suspensions were centrifuged and the supernatants saved.

The two supernatants were then tested for proteolytic activity by the method of Anson (1938). Four minutes after the Folin-Ciocalteau color reagent was added. the optical density, at 700 mμ, was determined with a Coleman spectrophotometer. The results of two experiments are given below; data are expressed in arbitrary units:

| | Preparation | Proteolytic activity |
|---|---|---|
| Experiment I | Frozen early | 11 |
| | Frozen late | − 2 |
| Experiment II | Frozen early | 19 |
| | Frozen late | − 2 |

While the experiments are only preliminary, some interesting possibilities are suggested. It appears that the extracts of the eggs that were frozen about 5 minutes after shedding have a substantial proteolytic activity. This activity would result, presumably, in part from activation before freezing and in part from activation of the dried preparation upon re-suspension in water—the calcium content of the egg material probably being sufficient to produce some protease activation. On the other hand those eggs which had been frozen about 14 minutes after shedding had virtually no activity—the negative figures may be insignificant. The lack of activity in this case can be regarded as the result of a natural protective mechanism of the cell, which would come into play to block further proteolytic digestion before it could act on components of the cell other than the nuclear membrane. This could prevent a possible autolysis. Heilbrunn (1952, p. 654) has considered this type of eventuality and suggests that the phenomenon may be the result of some sort of cellular homeostasis. Thus, in this particular instance it is possible that, following nuclear breakdown, the activated protease could liberate heparin, or a like substance, from binding to protein. (The feasibility of this mechanism has been demonstrated by *in vitro* experiments utilizing trypsin to free heparin from a complex with protamine (Kelly, 1951).) This heparin-like compound would then, presumably, be capable of blocking further proteolysis, and this idea finds support from the evidence showing that heparin is capable of preventing digestion of proteins by trypsin (Glazko and Ferguson, 1940). Further support of this hypothesis is offered by some *in vivo* observations of Kelly (1950). Kelly, by means of metachromatic staining with toluidine blue, has found that there are "granules" of heparin-like material present in Chaetopterus eggs following nuclear breakdown but he was unable to detect their presence before the dissolution of the nucleus (personal communication).

It has been difficult, thus far, to obtain a suitable extract of the eggs within the first minute after shedding. This is regrettable, since the theory holds that the eggs should have little or no proteolytic activity before calcium activation has occurred and it would be desirable to test this. Nevertheless, if we assume that the calcium-activated factor behaves as postulated here, it would not be conclusive proof that this was the system leading to nuclear breakdown in the cell. Direct proof would come from a demonstration that the isolated factor could act on its "natural" substrate—namely, the isolated nuclei. The few experiments that have been performed with this in mind have yielded no information since it has been impossible, as yet, to isolate the nuclei in any condition approaching a reasonably normal physiological state.

## Conclusions

From the evidence presented here, the following conclusions have been provisionally drawn regarding the process of activation and nuclear breakdown in the Chaetopterus egg:

1. Within the animal the eggs are "primed" for activation, but some inhibitor is present in the ovarian environment which presumably blocks the release of calcium from the protoplasmic cortex.

2. Following the liberation of the eggs from the animal, calcium ions are released —probably from binding with protein or lipoprotein in the cortex of the cell.

3. Within three minutes after the initial stimulus the free calcium ions activate a system, which is thought to involve a proteolytic enzyme.

4. This activated system then acts directly on the nucleus and produces a dissolution of the nuclear membrane.

5. Immediately following nuclear breakdown some protective mechanism comes into play to prevent excess autolysis of the cell, and probably to allow the cyclic events of mitosis to continue.

The above ideas probably represent the simplest possible mechanism that could be operating.

Two critical problems deserve the earliest consideration. First, it is desirable to achieve a better characterization of the calcium-activated system. Second, a verification of the hypothesis requires that we determine whether or not the isolated system will act on isolated nuclei.

## Summary

1. A study has been made of activation and nuclear breakdown in Chaetopterus eggs in an effort to elucidate the various steps in the response mechanism.

2. Within two minutes after the immature egg is stimulated there is a liquefaction of the protoplasmic cortex, which occurs as a prelude to the breakdown of the nucleus. The cortical change appears to be associated with a release of calcium ions from that region.

3. Within three minutes after the initial stimulus calcium ions activate a system which acts more directly on the nucleus and which, having once been activated, no longer requires the presence of calcium ions.

4. It was postulated that the system activated by calcium involved a proteolytic enzyme. To test this possibility experiments were run utilizing crystalline trypsin. It was found that trypsin could produce nuclear breakdown in the absence of calcium and that this activity was completely blocked by soybean trypsin inhibitor. Furthermore, when eggs were placed in trypsin solutions made up in acid media (pH 3.5–4.0), nuclear breakdown still occurred, indicating that the enzyme was penetrating into the interior of the cell.

5. Preliminary experiments suggest that there is a protease in the eggs which behaves as predicted. In addition, it appears that once nuclear breakdown is effected, some mechanism comes into play to block further action of the enzyme.

## LITERATURE CITED


ALLYN, H. M., 1912. The initiation of development in Chaetopterus. *Biol. Bull.*, **24**: 21–72.

ANSON, M. L., 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.*, **22**: 79–91.

BOHUS JENSEN, A., 1948. Activation of unfertilized sea urchin eggs by trypsin. *Nature*, **162**: 931–932.

CALLAN, H. G., 1952. A general account of experimental work on Amphibian oocyte nuclei. *Symposia Soc. Exp. Biol.*, **6**: 243–255.

DALCQ, A., 1928. Le rôle du calcium et du potassium dans l'entreé en maturation de l'oeuf de Pholade (*Barnea candida*). *Protoplasma*, **4**: 18–44.

DANIELLI, J. F., 1951. Chapter IV in: Cytology and Cell Physiology, G. H. Bourne, ed. Oxford University Press, Oxford.

GLAZKO, A. J., AND J. H. FERGUSON, 1940. Inhibition of tryptases by heparin. *Proc. Soc. Exp. Biol. Med.*, **45**: 43–46.

GOLDSTEIN, L., 1950. An experimental study of maturation in Chaetopterus eggs. *Biol. Bull.*, **99**: 341.

HEILBRUNN, L. V., 1952. An outline of general physiology. Third edition. W. B. Saunders Co., Philadelphia.

HEILBRUNN, L. V., AND K. M. WILBUR, 1937. Stimulation and nuclear breakdown in the Nereis egg. *Biol. Bull.*, **73**: 557–564.

HÖRSTADIUS, S., 1923. Physiologische Untersuchungen über die Eireifung bei *Pomatocerus triqueter L. Arch. mikroskop. Anat. Entw.*, **98**: 1–9.

KELLY, J. W., 1950. The localization of a metachromatic substance in the Chaetopterus egg. *Protoplasma*, **39**: 386–388.

KELLY, J. W., 1951. Effects of x-ray and trypsin on the metachromasy of heparin-basic protein combinations. *Biol. Bull.*, **101**: 223.

LEFEVRE, P. G., 1945. Certain chemical factors influencing artificial activation of Nereis eggs. *Biol. Bull.*, **89**: 144–156.

LILLIE, R. S., 1941. Further experiments on artificial parthenogenesis in starfish eggs, with a review. *Physiol. Zool.*, **14**: 239–267.

LINDAHL, P. E., 1937. Physiologisch-chemische Probleme der Embryonalentwicklung. *Fortschritte der Zoologie*, **3**: 271–305.

MAZIA, D., 1937. The release of calcium in Arbacia eggs on fertilization. *J. Cell. Comp. Physiol.*, **10**: 291–304.

MOSER, F., 1939. Studies on a cortical layer response to stimulating agents in the Arbacia egg. I. Response to insemination. *J. Exp. Zool.*, **80**: 423–445.

ÖRSTRÖM, A., AND M. ÖRSTRÖM, 1942. Über die Bindung von Kalzium in Ei und Larve von *Paracentrotus lividus. Protoplasma*, **36**: 475–490.

PASTEELS, J. J., 1935. Recherches sur le déterminisme de l'entrée en maturation de l'oeuf chez divers Invertébrés marins. *Arch. de Biol.*, **46**: 229–262.

RUNNSTRÖM, J., 1949. The mechanism of fertilization in metazoa. *Advances in Enzymology*, **9**: 241–327.

SCHEER, B. T., AND M. A. R. SCHEER, 1947. Some interrelationships of drug and ion actions in the artificial activation of marine eggs. *Physiol. Zool.*, **20**: 15–32.

SCOTT, A. C., AND G. LEBARON, JR., 1950. Factors involved in the breakdown of the germinal vesicle in the egg of *Chaetopterus pergamentaceus*. *Biol. Bull.*, **99**: 363.

TYLER, A., 1941. Artificial parthenogenesis. *Biol. Rev.*, **16**: 291–336.

WATTENBERG, H., 1938. Zur Chemie de Meerwassers. Über die in Spuren vorkommenden Elemente. *Zeitschr. f. anorg. u. allgem. Chem.*, **236**: 339–360.

WILSON, W. L., 1951. The rigidity of the cell cortex during cell division. *J. Cell. Comp. Physiol.*, **38**: 409–415.

WILSON, W. L., AND L. V. HEILBRUNN, 1952. The protoplasmic cortex in relation to stimulation. *Biol. Bull.*, **103**: 139–144.

YAMANE, J., 1930. The proteolytic action of mammalian spermatozoa and its bearing upon the second maturation division of ova. *Cytologia*, **1**: 394–403.

# PHOTOSYNTHESIS AND PHOTOTAXIS IN ULVA LACTUCA GAMETES[1]

F. T. HAXO AND K. A. CLENDENNING

*Division of Marine Botany, Scripps Institution of Oceanography, University of California, La Jolla, California, and Charles F. Kettering Foundation for the Study of Chlorophyll and Photosynthesis, Antioch College, Yellow Springs, Ohio*

During reproduction many multicellular algae form fertile areas which differ in color from the vegetative portions and from which are ultimately shed diversely pigmented gametes. These color differences may involve large changes in the ratios of the chlorophylls, carotenoids or phycobilins present in vegetative cells, making such algal material useful for studies concerned with the physiological functions of plastid pigments (Carter, Cross, Heilbron and Jones, 1948; Strain, 1951; Haxo and Blinks, 1950).

The physiology of algal gametes themselves is incompletely understood. For example, few observations have been reported concerning the phototaxis and respiratory activity of these specialized cells and the extent to which they have retained the capacity for photosynthesis. In *Fucus vesiculosus,* Whitaker (1931) reported that the olive-green, non-motile eggs are photosynthetically active, whereas the motile, orange antherozoids are inactive, or at best only very slightly active in this respect, but display a very high rate of oxygen consumption (about 5 times that of the unfertilized egg and about 13 times that of the vegetative tissue). Carter, Cross, Heilbron and Jones (1948 and personal communication, 1952) observed that beta-carotene is the predominating pigment of the orange antherozoids in three species of *Fucus* and in *Ascophyllum nodosum;* chlorophyll was not present in *Fucus vesiculosus* in sufficient amount to be either isolated or identified.

In various members of the Ulvales, the physiological and biochemical changes associated with gametogenesis and sporogenesis can be followed in the intact thallus and such studies can subsequently be extended to the free-swimming gametes. In the sea-lettuce, *Ulva lactuca,* the thallus in both diploid and haploid generations is a broad sheet of rather uniform thickness, composed of two layers of cells embedded in a gelatinous matrix. The gametophyte is heterothallic and during reproduction the vegetative cells give rise progressively to "chlamydomonad-type" gametes along the otherwise undifferentiated thallus margins (Schiller, 1907; Carter, 1926; Smith, 1947). It has been reported that division of the vegetative protoplast takes place at night, resulting in the formation of eight biflagellate gametes which are shed early in the morning (Schiller, 1907; Carter, 1926). After fusion of the isogamous gametes, there is a reversal of the light response, the zygotes being negatively phototactic.

[1] Contribution from the Marine Biological Laboratory, Woods Hole, Massachusetts, the Department of Biology, Johns Hopkins University, Baltimore, Maryland, and The Charles F. Kettering Foundation for the Study of Chlorophyll and Photosynthesis, Antioch College, Yellow Springs, Ohio.

The color differences associated with gamete formation in *U. lactuca* are apparently similar to, but not always as marked as, those reported by Smith (1947) for *U. lobata* found on the coast of California. In the latter species, the fertile portions of the female thalli are olive-green, and the corresponding portions of the male thalli are tan in color. Strain (1951) has attributed the color changes in the *U. lobata* to variations in the proportions of the chlorophylls and carotenes, the fertile portions of the thalli containing five times as much carotene as the grass-green portions. Smith (1947, 1951) reported that *U. lobata* shows a definite fortnightly periodicity in gamete formation and liberation which is correlated with tidal succession. At Woods Hole, Mass., during the summer months fertile *U. lactuca* can usually be found only at intervals at any one location, suggesting a periodic reproduction; however, the extent to which reproduction in this species is under environmental control is not known.

The present investigation is concerned with studies of photosynthesis, respiration, phototaxis, and pigmentation in free-swimming gametes of *U. lactuca*, as well as some corresponding observations on vegetative thalli and fruiting thallus margins.

## Materials and Methods

Fronds of *Ulva lactuca* L. var. *rigida* Le Jolis (*cf.* Taylor, 1937) which, by the characteristic opacity and color of the thallus margins, showed evidence of incipient reproduction were collected every few days at low tide in the vicinity of Woods Hole during July and August, 1951. They were maintained in individual pans of sea water in the laboratory until the reproductive cells were shed. Not infrequently a fair percentage of the thalli treated in this manner failed to release either gametes or zoospores. Most fronds which did shed were found to be gametophytic, a distinction which could not be made in the field since the fruiting margins of both female gametophytes and sporophytes are similar in color. The gametes were concentrated by phototaxis, collected with an eye-dropper, and in some cases tested for sex according to the method employed by Smith (1947). The gametes were transferred to centrifuge tubes for measurement of packed cell volume.

Comparison of the rates of photosynthesis and respiration of vegetative thalli and unshed fruiting margins were made on freshly cut discs and strips of thallus. Manometric measurements were made at 25° C. in artificial or natural sea water containing 0.016 *M* carbonate-bicarbonate and having an initial pH of 8.5 (Clendenning and Haxo, unpublished). The light source was a 1000 watt air-cooled projection lamp which provided a maximum light intensity of 5400 f.c. at the manometer vessel. The light was filtered through eighteen inches of water. Polarographic measurements of oxygen production were made in saturating light at about 25° C. by the procedure described by Haxo and Blinks (1950).

Phototactic movement was measured in a cylindrical absorption cell (thickness 1.0 cm., diameter 2.5 cm.) provided externally with a vertical cross hair. By alternate illumination of opposite sides of the cuvette, the gametes which had been introduced as a dilute suspension were first collected as a very thin and uniform layer on the glass wall, and then made to cross the cuvette as a sharply defined wave. The time taken for the wave of gametes to move 5 mm. to the midpoint of the cuvette, as determined visually, was taken as a measure of the motility rate. Rate of movement in different spectral regions of equal intensity was measured

with the aid of an Eppley thermopile and Farrand interference filters having peak transmissions at about 440, 560, 620, 680 and 700 mμ. White light intensities were measured with a Weston Sunlight Illumination Meter.

The chloroplast pigments were extracted by one-minute exposure to hot water followed by repeated extractions with methanol. Extraction with absolute methanol was continued until the samples were colorless. The extracted pigments were transferred to ethyl ether by the addition of concentrated sodium chloride solution. Water was removed from the ether by chilling and subsequent drying with anhydrous sodium sulfate. The chlorophyll was estimated spectrophotometrically (Model B Beckman) on an aliquot of the total pigment solution, using Comar's equations (1942). The carotenoids were separated from the chlorophyll by saponifying the total pigment extract with 10–20% KOH. Carotenes and xanthophylls were separated by partition of the petroleum ether solution over 90% methanol and were estimated spectrophotometrically in petroleum ether. Total carotenoids were also estimated from extinction measurements made at 445 mμ on the total pigment extract after correcting for chlorophyll absorption at this wave-length. The total carotenoid values obtained in this way were in close agreement with the sums of the separately determined carotenes and xanthophyll contents. The carotenes from the male gametes were fractionated chromatographically on a column of powdered magnesium oxide and identified on the basis of their characteristic absorption spectra in hexane. For purposes of standardization, alpha- and beta-carotene were isolated from carrots and their absorption curves determined with the spectrophotometer employed in this investigation.

## Results

The photosynthetic and respiratory activities of equal areas (1.77 cm.$^2$) of vegetative and fruiting portions of *Ulva* thallus which were observed manometrically are shown in Figure 1. Rectangular Warburg vessels containing 7 ml. of bicarbonate-enriched sea water (0.016 *M*) were employed in this experiment. This volume of liquid provided a large reservoir of bicarbonate, but the associated diffusion lag obscured the details of the transitions. Rates calculated from these data refer to the steady state (Table I). Respiration was much higher in the fruiting thallus margins than in the green vegetative tissue, whereas both net and total photosynthesis was lower. The photosynthetic and respiratory activities are expressed in different terms in Table I, which allow comparisons with the data for free-swimming gametes presented in Table III.

The early time course of photosynthesis in vegetative *Ulva* and other aquatic plants has been previously characterized polarographically by Blinks and Skow (1938), the essential features being an initial oxygen gush, followed by an induction period before the main trend of oxygen evolution resumes. In the present experiments fruiting thallus margins consistently exhibited a longer induction period and lower final rates of $O_2$ production than vegetative parts of the same thallus after identical pre-treatments. In a typical experiment the tissue was held tightly against the electrode with cellophane as in earlier applications, a condition which would magnify the effects of a high respiration rate on photosynthetic induction, and was maintained in dim light for one hour prior to illumination with saturating light. Oxygen production by the fruiting thallus was sluggish and

detectable only in the third minute of illumination. Successive light exposures reduced, but did not abolish, the induction period (Fig. 2a, b, c, d) and no suggestion of an oxygen gush was observed. In the vegetative tissue, however, the oxygen gush and induction period were complete within the first 30 seconds and, upon successive exposures to strong light, oxygen production proceeded at its full rate almost instantaneously, in agreement with the earlier findings of Blinks and Skow (1938, Fig. 5; *cf.* Haxo and Blinks, 1950, Fig. 6).

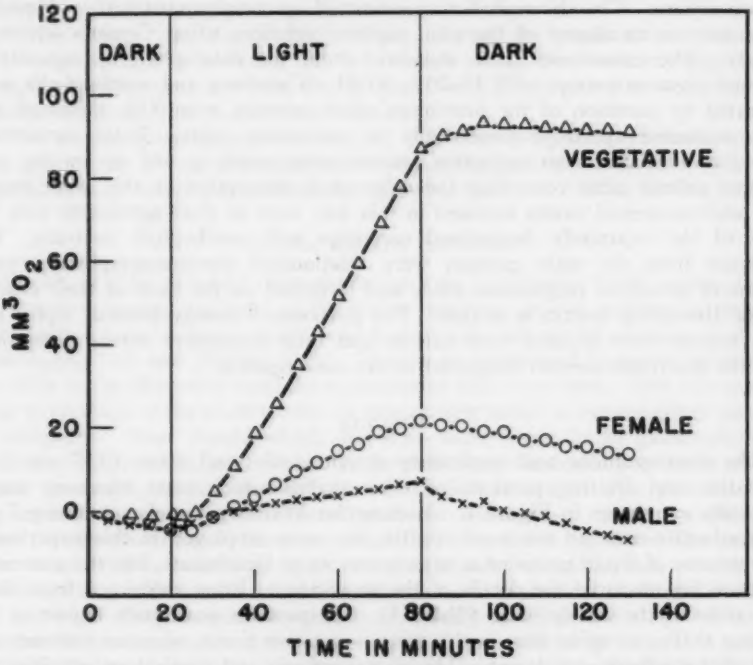



FIGURE 1. Photosynthesis and respiration of vegetative tissue and fruiting thallus margins in carbonate-enriched sea water at 25° C. Area of thallus disc, 1.77 cm².

The tissues employed in the foregoing manometric experiment were analyzed for solids, chlorophyll and carotenoids (Table II). The yellowish or male fruiting thallus margin showed the lowest chlorophyll content. The olive-green fruiting margins (in all probability female, but mating responses were not determined in this instance) contained about the same chlorophyll concentration as the vegetative thallus. The carotene contents of both types of fruiting thallus margins were about three times higher than that of the vegetative thallus. The xanthophyll contents of all three samples were essentially the same, so that there was more xanthophyll than carotene in the vegetative tissue, and less xanthophyll than carotene in the reproductive thallus margins. The pigment analyses for the vegetative thalli are

TABLE I

*Photosynthetic and respiratory activity of vegetative thallus and fruiting thallus margins of Ulva lactuca*

| Gas exchange | Fruiting thallus margin | | Vegetative thallus |
|---|---|---|---|
| | Male | Female | |
| Photosynthesis (corrected) | | | |
| mm.³ $O_2$/hr./dm.² | 1290 | 2100 | 5980 |
| mm.³ $O_2$/hr./gm. fresh wt. | 1620 | 3070 | 6180 |
| mm.³ $O_2$/hr./gm./solids | 7110 | 12,300 | 25,400 |
| mm.³ $O_2$/hr./mg. chlorophyll | 1320 | 1720 | 3490 |
| Respiration | | | |
| mm.³ $O_2$/hr./dm.² | 755 | 605 | 270 |
| mm.³ $O_2$/hr./gm. fresh wt. | 950 | 885 | 280 |
| mm.³ $O_2$/hr./gm. solids | 4180 | 3530 | 1180 |
| mm.³ $O_2$/hr./mg. chlorophyll | 775 | 499 | 160 |

in reasonable agreement with those reported previously for *U. lactuca* by Seybold and Egle (1938). These authors gave the following values (when expressed as mg./gm. solids): chlorophyll-a, 3.3; chlorophyll-b, 1.5; total chlorophyll, 4.8; carotene, 0.16; xanthophyll, 0.77; total carotenoid, 0.93.

Expressed on a solids basis, the free-swimming female gametes contain twice as much chlorophyll and half as much carotene as the male gametes; the xanthophyll content was the same in both cases (Table III). Comparisons cannot readily be made on the same basis between the pigment content of the free-swimming gametes and that of the vegetative thalli (Table II). It is interesting to note, however, that the ratio of chlorophyll:carotenoid has changed from 8.3 in the vegetative tissue to 2.7 in the female gametes and 1.1 in the male gametes. Strain (1951) has reported that in *U. lobata* a large portion of the increased carotene content of the sexually differentiated margins is due to the gamma-carotene isomer, only a little of which occurs in vegetative thalli. A similar change appears to occur in *U. lactuca*.

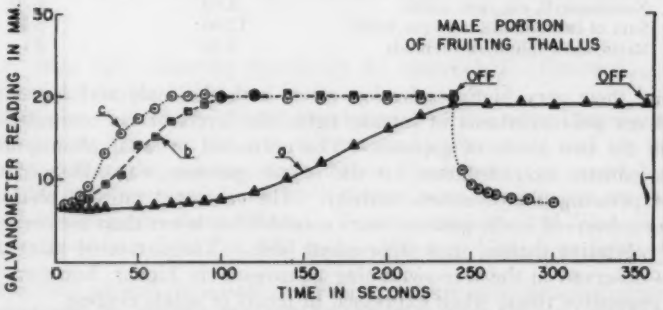



FIGURE 2. Initial time course of oxygen evolution which was observed polarographically on a strip of male fruiting thallus margins at *ca.* 25° C., after a pre-treatment of one hour in dim light.

TABLE II

*Chlorophyll, carotenoid and solids data for vegetative and reproductive thallus margins of Ulva lactuca*

| Constituents | Fruiting thallus margins | | Vegetative thallus |
|---|---|---|---|
| | Yellowish | Greenish | |
| Fresh wt., mg./dm.[1] | 797 | 683 | 967 |
| Solids, mg./dm.[2] | 181 | 171 | 230 |
| Chlorophyll, mg./gm. solids | 5.41 | 7.15 | 7.29 |
| Carotene, mg./gm. solids | 1.35 | 1.49 | 0.34 |
| Xanthophyll, mg./gm. solids | 0.68 | 0.64 | 0.54 |
| Sum of carotenoids, mg./gm. solids | 2.03 | 2.13 | 0.88 |
| Ratio, chlorophyll/carotenoids | 2.7 | 3.4 | 8.3 |

Chromatographic and spectral analyses of the abundant carotene fraction present in the male gametes indicated the following composition: 43% gamma-carotene, 32% beta-carotene, 3% alpha-carotene, and 22% unidentified carotene.

The green (♀) and yellow (♂) gametes, whose photosynthesis and respiration are reported in Figure 3 and in Table IV, were actively phototactic at the time, and also showed the normal clumping reaction of opposite sexes on admixture. Respiration of the male gametes exceeded that of the female,[2] and respiration of both types exceeded that of the unshed fruiting margin as well as of the vegetative thallus. Expressed on a solids basis, the respiration of the male gametes was almost thirty times greater than that of the vegetative thallus, and was about fourteen times greater in terms of chlorophyll content.

TABLE III

*Chlorophyll, carotenoid and solids data for Ulva lactuca gametes*

| Constituents | Male gametes | Female gametes |
|---|---|---|
| Dry wt., mg./ml. packed cells | 96 | 105 |
| Chlorophyll, mg./gm. solids | 14.42 | 24.0 |
| Carotene, mg./gm. solids | 8.20 | 4.68 |
| Xanthophyll, mg./gm. solids | 4.70 | 4.20 |
| Sum of carotenoids, mg./gm. solids | 12.90 | 8.88 |
| Ratio, chlorophyll/carotenoids | 1.12 | 2.7 |

Despite their very high respiration rates, both the male and female gametes exhibited net photosynthesis in intense light, the levels of net assimilation being similar in the two kinds of gametes. The corrected or total photosynthesis for the male gametes exceeded that for the female gametes, regardless of the basis used in expressing photosynthetic activity. The corrected rates of photosynthesis which were observed in the gametes were considerably lower than the corresponding rate for vegetative thallus on a chlorophyll basis. The corrected rates of photosynthesis observed in the free-swimming gametes were higher, however, than the rate for vegetative tissue when expressed in terms of solids content.

[2] The present data are insufficient to establish whether the isogamous male and female gametes of *U. lactuca* differ significantly in this respect.

Photosynthesis by the gametes has been compared manometrically in red and white light (Fig. 4). The rate of oxygen evolution obtained with saturating white light was identical to that observed on exposure of the gametes to the component red light. In contrast to their phototaxis, photosynthesis by the gametes was effectively sensitized by light absorbed specifically by chlorophylls. Their photosynthetic mechanism could be completely saturated by red light which was entirely ineffective in orienting the swimming motion. This experiment does not establish the relative effectiveness of chlorophylls and carotenoids as sensitizers of photosynthesis in the gametes of green algae.[3] However, all earlier studies of vegetative green algae have indicated that light absorbed by the carotenoids is utilized less

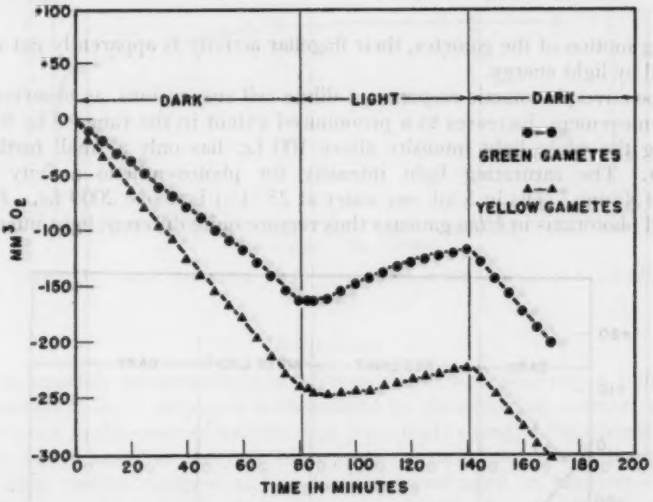



FIGURE 3. Photosynthesis and respiration in male and female *Ulva* gametes in carbonate-enriched sea water at 25° C.; 0.0815 mg. chlorophyll in male, 0.161 mg. chlorophyll in female gametes.

effectively than light absorbed specifically by chlorophyll (Emerson and Lewis, 1943; Haxo and Blinks, 1950).

*Ulva* gametes swim actively in total darkness as well as when illuminated. Thus when gametes are drawn to the side of a cuvette by phototaxis, and are then maintained in darkness, the gametes soon diffuse throughout the vessel by random motion. The same unoriented motility is shown when the gametes are illuminated by light of wave-lengths above 550 mμ. Positive phototaxis of *Ulva* gametes is a response to blue-violet light (ca. 440 mμ). Although blue-violet light orients the

[3] Unsuccessful attempts were made to determine the photosynthetic action spectra of male margins, employing the available polarographic apparatus which did not permit estimation of the large respiration correction. Upon illumination of this tissue at low and moderate light intensities, either no change in oxygen concentration at the electrode or an actual uptake of oxygen was noted.

TABLE IV

*Photosynthetic and respiratory activity of Ulva lactuca gametes*

| Gas exchange | Male gametes | Female gametes |
|---|---|---|
| Photosynthesis (corrected) | | |
| mm. $O_2$/hr./ml. packed cells | 3960 | 3550 |
| mm. $O_2$/hr./gm. solids | 41,200 | 33,800 |
| mm. $O_2$/hr./mg. chlorophyll | 2570 | 1410 |
| Respiration | | |
| mm.$^3$ $O_2$/hr./ml. packed cells | 3380 | 2750 |
| mm.$^3$ $O_2$/hr./gm. solids | 35,200 | 26,200 |
| mm.$^3$ $O_2$/hr./mg. chlorophyll | 2200 | 1090 |

swimming motion of the gametes, their flagellar activity is apparently not otherwise controlled by light energy.

The positive phototactic response of dilute cell suspensions, as observed by rate of linear movement, increases to a pronounced extent in the range 0 to 50 f.c., but increasing the white light intensity above 100 f.c. has only a small further effect (Fig. 5). The saturating light intensity for photosynthetic activity in *Ulva* gametes (50 mm.$^3$ cells in 5 ml. sea water at 25° C.) is above 2000 f.c. Photosynthesis and phototaxis in *Ulva* gametes thus require quite different light intensities for

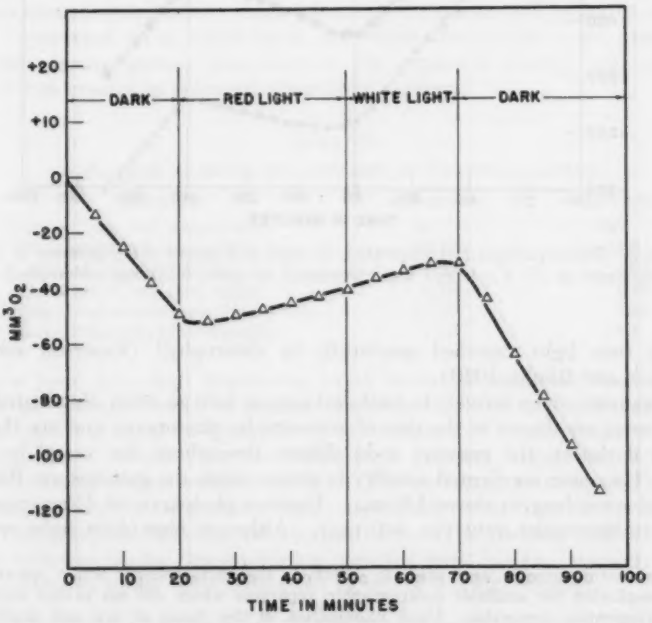



FIGURE 4. Photosynthesis and respiration in female *Ulva* gametes in red and white light, approximately 50 mm$^3$. gametes in 7 ml. carbonate-enriched sea water at 25° C.

saturation, and also have very different wave-length specificities. Red light is highly effective in sensitizing their photosynthesis although it plays no part in inducing the phototactic response.

The fastest rate at which *Ulva* gametes have been observed to swim towards a strong light source is *ca.* 18 mm. per minute (Fig. 4). The size of the gametes, exclusive of flagella, is *ca.* 7.6 by 3.6 $\mu$ (length 6.9–8.3 $\mu$, width 3.5–3.8 $\mu$). From the observed rates of phototactic movement and size of the cells, it is apparent that *Ulva* gametes swim more than two thousand times their body length in one minute.

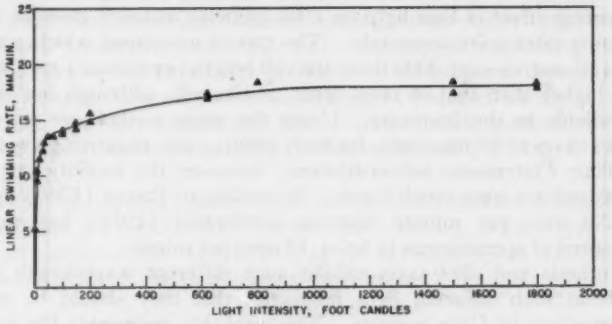



FIGURE 5. Maximum linear movement of *Ulva* gametes at *ca.* 25° C. as a function of light intensity.

## DISCUSSION

The manometric measurements of photosynthesis and respiration indicate that gamete formation in *U. lactuca* is accompanied by physiological changes which are detectable prior to the onset of motility and the actual release of the gametes. Most notable is the increase in the rate of oxygen consumption, which begins in the differentiating thallus margins and is further accentuated in the free-swimming gametes. It would be of interest to ascertain whether this increase in metabolic activity is gradual or abrupt, and whether it is associated with specific stages in gametogenesis. Although the entire vegetative thallus of the *Ulva* gametophyte may ultimately give rise to gametes, at any one time gametogenesis is normally restricted to the peripheral margins of the thallus. Fruiting specimens have been collected, however, in which the thallus has been torn internally, leaving the periphery intact. The vegetative area bordering the cut surfaces occasionally has been observed to have undergone color differentiation and ultimately to release gametes. Experimental studies might reveal more fully the conditions under which gamete formation is initiated and maturation of the gametes proceeds.

The pronounced induction period which was observed polarographically in the fruiting thallus margin is attributed to oxygen deficit resulting from the high respiration rate of this tissue, as well as its relative inaccessibility to oxygen while pressed tightly against the platinum electrode. Blinks and Skow (1938) had earlier found for vegetative *Ulva* and other aquatic plants that the induction loss is magnified by lengthy dark anaerobic periods, and may be eliminated by adequate aeration in the dark or by successive exposures to bright light.

The respiration of free-swimming *Ulva* gametes is very much higher than has been observed previously in photosynthetically active cells, and is about 50% higher than that reported by Whitaker for the non-photosynthetic *Fucus* antherozoids. The compensation point of *Ulva* gametes has not yet been determined accurately over a range of temperature and cell densities, but with 50 mm.$^3$ cells in 5 ml. sea water at 25° C., the compensating white light intensity was above 1500 f.c. Because of their high respiration rate, the compensation point for *Ulva* gametes is much higher than any of the values which were recently assembled by Rabinowitch (1951).

The orienting effect of blue light on *Ulva* gametes makes it possible to measure their swimming rates quite accurately. The rate of movement which was observed in this way (18 mm. or over 2000 times the cell length per minute) on *Ulva* gametes is probably higher than that of most other motile cells, although few data of this sort are available in the literature. Using the same method we have observed motility rates of up to 16 mm./min. for both positive and negative phototaxis in the green flagellate, *Platymonas subcordiformis;* however, the motility rates observed for several flagellates were much lower. According to Patten (1946) spermatozoa travel 1.5–3.0 mm. per minute whereas Rothschild (1951) has reported the translatory speed of spermatozoa to be *ca.* 12 mm. per minute.

Photosynthesis and phototaxis exhibit such different wave-length specificities and saturate at such different light intensities that they should be regarded as unrelated processes in *Ulva* gametes. This probably represents the situation for flagellated algae in general, since phototaxis in, for example, *Euglena* and *Chlamydomonas* (Mast, 1917), *Volvox* (Laurens and Hooker, 1920), and in the green and red phenotypes of *Dunaliella salina* (Blum and Fox, 1933) has been shown to be limited to the blue end of the spectrum. *Rhodospirillum rubrum* presents a special case since Thomas and Nijenhuis (1950) have reported that the action spectra, saturating light intensities and responses to cyanide and ethyl urethane were the same for photosynthesis and phototaxis in this organism.

Both male and female fertile portions of *U. lactuca* thalli contained more carotene than did vegetative portions of the thallus, being apparently similar in this respect to *U. lobata* (Strain, 1951). In the free-swimming gametes the carotene content of the male gametes was about twice that of the female reproductive cells. High carotenoid concentrations are generally characteristic of male gametes, whereas there is a greater retention of chlorophyll in the female gametes (Karrer *et al.*, 1943; Carter *et al.*, 1948; Strain, 1951). The widespread accumulation of carotenoids in reproductive cells and tissues suggests that carotenoids may play a role in sexual reproduction (Emerson and Fox, 1940; Cook, 1945; Goodwin, 1950), in addition to their probable role as sensitizers of phototaxis (Wald, 1943). Whatever the role of carotenoids in sexuality may be, it seems evident that the abundant accumulation sometimes observed in gametes is in considerable excess of the requirements for this function and may not be related to it. *Allomyces* species accumulate abundant stores of gamma-carotene in the male gametes (Emerson and Fox, 1940); however, Turian (1952) has inhibited the visible production of pigment without apparently altering the capacity for mating. The apparently normal sexual reproduction of albino strains of *Neurospora* (Hungate, 1945) is another case in point.

Photosynthetically active cells always contain at least traces of chlorophyll but the minimal chlorophyll requirements for photosynthesis have not been established with certainty (*cf.* Rabinowitch, 1945, 1951). In investigations of this question, advantages are presented by algal gametes, in that the cells are in a healthy state of high metabolic activity irrespective of their chlorophyll content. Furthermore, by use of several species it might be possible to obtain for study a series containing successively smaller amounts of chlorophyll. In *U. lactuca* the chlorophyll content of the female gametes was almost twice that of the male gametes and yet showed a lower photosynthetic activity in terms of chlorophyll content. A visible color differentiation of about the same degree is found in the gametes of *Bryopsis corticulans* but Blinks (personal communication, 1952) has observed that only the green (female) gametes show photosynthetic activity in excess of the compensation point. As was indicated previously, *Fucus* eggs contain chlorophyll and effect net photosynthesis in strong light, whereas, apparently, *Fucus* antherozoids contain no chlorophyll (Carter *et al.*, 1948) and are incapable of photosynthesis (Whitaker, 1931).

Thanks are due various colleagues in the Botany Division of the Marine Biological Laboratory for help in the collection and handling of the algae used in this study; and in particular Dr. Maxwell Doty for his suggestions and Shirley Trefz for assistance in some of the preliminary experiments.

## Summary

1. Fruiting margins of *Ulva lactuca* thallus are characterized by a higher content of carotenoids, as well as by higher respiratory and lower photosynthetic activity, than the corresponding vegetative thallus. Photosynthesis in the male margin was also characterized by a longer induction period than was observed in the vegetative thallus by the polarographic method.

2. The changes in pigment composition observed in *Ulva lactuca* during reproduction are similar to those reported for *Ulva lobata*. The male gametes of *Ulva lactuca* contained twice as much carotene and half as much chlorophyll as the female gametes. The male carotene fraction contained 43% gamma-carotene, 32% beta-carotene, 3% alpha-carotene and 22% unidentified carotene. The xanthophyll contents of the male and female gametes were similar. The chlorophyll/carotenoid ratio was 8.3 in the vegetative thallus, 2.7 in the female gametes and 1.1 in the male gametes.

3. The respiration of the male gametes exceeded that of the female gametes, was almost thirty times greater than that of the vegetative thallus on a solids basis, and was about twelve times greater in terms of chlorophyll content.

4. Both male and female gametes exhibited net photosynthesis in intense white light, but a very high light intensity was required to compensate their respiration, which is the highest that has been reported for cells possessing photosynthetic activity.

5. Positive phototaxis of *Ulva* gametes is a response to blue-violet light, which orients their swimming but does not otherwise control their flagellar activity. Their maximum rate of linear movement is attained at moderate light intensity (*ca.* 100 f.c.). The gametes swim 2500 times their own body length each minute.

## LITERATURE CITED


BLINKS, L. R., AND R. K. SKOW, 1938. The time course of photosynthesis as shown by a rapid electrode method for oxygen. *Proc. Nat. Acad. Sci.*, **24**: 413–419.

BLUM, H. F., AND D. L. FOX, 1933. Light responses in the brine flagellate *Dunaliella salina* with respect to wave length. *Univ. Calif. Pub. Physiol.*, **8**: 21–30.

CARTER, N., 1926. An investigation into the cytology and biology of the Ulvaceae. *Ann. Bot.*, **40**: 665–689.

CARTER, P. W., L. C. CROSS, I. M. HEILBRON AND E. R. H. JONES, 1948. The Algae IV. Lipochromes of the male and female gametes of some species of the Fucaceae. *Biochem. J.*, **43**: 349–352.

COMAR, C. L., 1942. Analysis of plant extracts for chlorophylls a and b. *Industrial and Engineering Chem.*, **14**: 877–879.

COOK, A. H., 1945. Algal pigments and their significance. *Biol. Rev.*, **19**: 115–132.

EMERSON, RALPH, AND D. L. FOX, 1940. γ-carotene in the sexual phase of the aquatic fungus *Allomyces*. *Proc. Royal Soc. London*, **128**: 275–293.

EMERSON, ROBERT, AND C. M. LEWIS, 1943. The dependence of quantum yield of *Chlorella* photosynthesis on wavelength of light. *Amer. J. Bot.*, **30**: 165-178.

GOODWIN, T. W., 1950. Carotenoids and reproduction. *Biol. Rev.*, **25**: 391–413.

HAXO, F. T., AND L. R. BLINKS, 1950. Photosynthetic action spectra of marine algae. *J. Gen. Physiol.*, **33**: 389–422.

HUNGATE, M. V., 1945. A genetic study of albino mutants of *Neurospora crassa*. Master's Thesis, Stanford University.

KARRER, P., W. FATZER, M. FAVARGER AND E. JUCKER, 1943. Die Antheridienfarbstoffe von Chara-Arten (Armleuchtergewachse). *Helv. Chim. Acta*, **26**: 2121–2122.

LAURENS, H., AND H. D. HOOKER, 1920. Studies on the relative physiological values of spectral lights. II. The sensitivity of *Volvox* to wave-lengths of equal energy content. *J. Exp. Zool.*, **30**: 345–368.

MAST, S. O., 1917. The relation between spectral color and stimulation in the lower organisms. *J. Exp. Zool.*, **22**: 471–528.

PATTEN, B. M., 1946. Human embryology. P. Blakiston Co., Phila.

RABINOWITCH, E. I., 1945, 1951. Photosynthesis and related processes, Vol. I, IIa. Interscience Publishers Inc., New York.

ROTHSCHILD, LORD, 1951. Sea-urchin spermatozoa. *Biol. Rev.*, **26**: 1–27.

SCHILLER, J., 1907. Beiträge zur Kenntnis der Entwicklung der Gattung *Ulva*. *Sitzungsber. Akad. Wiss. Wien (Math.-Nat. Kl.)*, **116–1**: 1691–1716.

SEYBOLD, A., AND K. EGLE, 1938. Quantitative Untersuchungen über Chlorophyll und Carotinoide der Meeresalgen. *Jahrb. Wiss. Bot.*, **86**: 50–80.

SMITH, G. M., 1947. On the reproduction of some Pacific Coast species of *Ulva*. *Amer. J. Bot.*, **34**: 80–87.

SMITH, G. M., 1951. Sexuality of algae. Manual of Phycology. G. M. Smith, ed. Chronica Botanica, Waltham, Mass.

STRAIN, H., 1951. The pigments of algae. Manual of Phycology. G. M. Smith, ed. Chronica Botanica, Waltham, Mass.

TAYLOR, W. R., 1937. Marine algae of the northeastern coast of North America. Univ. of Mich. Press.

THOMAS, J. B., AND L. E. NIJENHUIS, 1950. On the relation between phototaxis and photosynthesis in *Rhodospirillum rubrum*. *Biochim et Biophys. Acta*, **6**: 317–324.

TURIAN, G., 1952. Carotenoides et differenciation sexuelle chez *Allomyces*. *Experientia*, **8**: 302.

WALD, G., 1943. The photoreceptor functions of the carotenoids and vitamin A. Vitamins and Hormones, R. Harris and K. V. Thimann, eds., **1**: 195–227.

WHITAKER, D. M., 1931. On the rate of oxygen consumption by fertilized and unfertilized eggs. I. *Fucus vesiculosus*. *J. Gen. Physiol.*, **15**: 167–182.

# A STUDY OF CHEMORECEPTION IN AQUEOUS AND GAS PHASES[1]

EDWARD S. HODGSON

*Dept. of Zoology, Barnard College, Columbia University, New York, New York*

Although it is generally recognized that chemoreception plays an important part in the orientation, feeding and reproductive activities of many aquatic animals (Jahn and Wulff, 1950; Walker and Hasler, 1949), these animals have been a perplexing group in developing concepts of the physiology of the chemical senses. At first it was even questioned whether presumed chemical sense organs of aquatic organisms could have any function at all, since they were surrounded by a fluid medium rather than a gaseous one (Nagel, 1894). Nagel's negative conclusion, based upon anthropomorphic considerations, has long been discredited by experiments indicating that many reactions of aquatic animals are mediated by receptors which he held to be functionless—*e.g.*, the receptors in the olfactory pits and connected to the olfactory nerves (Parker and Sheldon, 1913; von Frisch, 1924).

Matthes (1924), interested in adaptations of the sensory apparatus to different environments, then raised the question whether the same receptors could mediate reactions to chemical stimuli underwater and on land in the case of the animals which spent a portion of their life cycles in each environment. It was found that *Triton* no longer reacted to chemicals in either environment when the olfactory nerve was cut. The olfactory innervation was traced to the Jacobson's organ (Matthes, 1927) and it was shown that the presumed receptors in the larval and adult animals were histologically very similar to those of other vertebrates. When it was discovered that a sheet of mucus, variable in quantity and distribution according to the physiological state of the animal, overlay the olfactory receptors (Murphy, 1931; Leasure, 1939) the logical assumption that chemical stimuli went into solution in the mucus layer abridged the usefulness of the previous observations on vertebrates for understanding what specializations, if any, of the receptors existed for functioning in gas and liquid phases. Schaller (1926) avoided this difficulty by studying amphibious beetles which have chemoreceptors on mouthparts accessible for ablation experiments, and which could be tested both underwater and with a dry cuticular surface in air. Conditioned reactions of *Dytiscus* to coumarin and synthetic musk, either underwater or in air, ceased after removal of the flagellar portion of the antennae and the maxillary palpi. Acid, salt, and sugar were similarly detected by receptors on the labial palpi and the inside of the mouth. It was concluded that different groups of receptors were specialized for perception of either of two types of chemical stimuli—those which are "odor-substances" for man (irrespective of physical state of the stimulus), or those compounds which are

[1] The author takes pleasure in acknowledging his indebtedness to Dr. V. G. Dethier of the Johns Hopkins University for the loan of some of the equipment used in this work, and for his critical reading of the manuscript.

"taste-substances" for man, with secondary specializations for perceiving particular modalities of taste substances, such as acid, salt, sugar, and bitter compounds, since these compounds are discriminated by *Dytiscus*. However, conflicting conclusions based upon experiments with the same and related species of insects were presented by Ritter (1936) and Bauer (1938). Although accepting Schaller's main concept about the types of physiological specializations to be found in chemoreceptors, they did not agree on the location or morphology of the receptor groups sensitive to "odor-substances," "taste-substances," or the various modalities of the latter group. Ritter (1936) reported that the antennae of *Hydrous* completely lacked chemoreceptors and that heliotropin and skatol stimulated receptors on the tips of the maxillary palpi; the receptors of the labial palpi were reported to be sensitive only to acid. Bauer (1938) failed to prevent reaction to non-acidic stimuli by removing the maxillary palpi. Nor was agreement reached on the morphological appearance of the variously located and specialized receptors.

In a recent review, Dethier and Chadwick (1948) discussed possible sources of conflicting data in the experiments cited above (such as the lack of quantitative control of the stimulus) and have concluded that the relationship between the physical state of a chemical and its effect on chemoreceptors is unknown. They further conclude that this relationship should be explored not only to understand what specializations, if any, differentiate receptors functioning in gas and liquid phases and the sensitivity to various modalities of stimuli in each phase, but also to determine whether the same limiting mechanism operates in both phases. The aim of the present work is to determine, first of all, whether the same set of receptors mediates reactions to chemical stimuli administered in gas and liquid phases and whether different groups of receptors are specialized for perception of particular modalities of stimuli in either phase. The question of the fundamental limiting mechanism in each phase is later discussed.

## Materials and Methods

The experimental animal was an amphibious beetle, *Laccophilus maculosus* Germar, chosen because of its dual air-water habitat, accessible mouthparts, and availability. It has been shown (Hodgson, 1951) that *Laccophilus* possesses much lower reaction thresholds to a wide variety of chemical stimuli than those reported for other insects. Since the relations between molecular structure, chemical properties, and relative stimulating effectiveness of compounds included in several series of organic and inorganic chemicals determined for *Laccophilus* are essentially identical with the findings obtained in experiments with *Phormia* (Dethier and Chadwick, 1948), *Balanus* (Cole and Allison, 1930) and other terrestrial and aquatic invertebrates, it was concluded that at least the limiting mechanisms of chemoreception in *Laccophilus* were not aberrant and might yield information of general significance. The beetles were collected at the Fish Hatcheries Experiment Station of the United States Fish and Wildlife Service at Leetown, West Virginia. They were obtained in large numbers both in shallow water of the fish rearing ponds and in dry grass near the ponds. The methods used for maintaining stocks of these animals have been previously described (Hodgson, 1951). Every precaution was taken to standardize feeding and handling of the beetles and after being once used in the experiments the beetles were discarded.

The technique for determining threshold concentrations of pure chemicals administered in the gas phase or in the liquid phase is essentially the same, although a different apparatus is required for each of the two types of test. The over-all procedure is to apply moving, unmixed streams of air or water, containing known concentrations of the chemical to be tested, to groups of beetles and determine the percentage of beetles which react to the chemical by moving into the control area of the apparatus which is exposed to a moving stream of air or water identical in all respects except that the test compound is absent. Technical details of the construction and operation of the apparatus for determining threshold concentrations of chemicals in aqueous solution have been fully presented (Hodgson, 1951) and hence will not be repeated, since the method used here was identical. The apparatus for determining threshold concentrations of gases was a slight modification of the olfactometer described in detail by Dethier and Yost (1952), and used by these investigators in experiments with the blowfly, *Phormia*. In brief summary of this method, animals to be tested are placed in a cage through which pass two streams of air, one containing a known concentration of the gaseous chemical being tested. Before the experiment, the animals are distributed randomly in the two halves of the cage exposed to the experimental and control streams of air. Those reacting to the gas being tested move to the control side of the cage. The percentage doing so is determined by photographs taken during the run. With *Laccophilus*, it was found that the best results were obtained using a smaller cage (4″ × 2″ × 3/8″) than was used with *Phormia*. This narrow cage was placed horizontally in the apparatus so that the air stream passed through it from top to bottom, since *Laccophilus* did not climb the vertical walls of the cage as readily as did *Phormia*. Fifty unanesthetized beetles, completely dry on the external surface of the cuticle, were placed in the cage at one time. Since the number of beetles reacting is equal to the difference between the average number of beetles remaining on the experimental side of the cage and the average number originally on that side (about 25 beetles), the formula used to obtain the percentage reaction to any particular concentration was as follows: $\%\ \text{reaction} = \frac{25 - x}{25}$, where $x$ is the number of beetles remaining on the exposed side of the cage. Aside from the slight modification of the test cage construction, this procedure was identical with that described by Dethier and Yost (1952), and their report may be consulted for technical details of the apparatus, sample calibration curves, etc.

With either aqueous or gas phase tests, doubling concentrations of the chemical stimulus are administered. The percentage of beetles reacting at each concentration is converted into probit units by the method of Bliss (1938). This conversion merely transforms the typical sigmoid dosage-reaction curve to a straight line, in which the 50% reaction point corresponds to a probit of 5. The direct relationship between the logarithm of the molar concentration of the stimulant and the percentage of beetles reacting is shown in Figure 1. The data used in this plot were selected simply because the thresholds in these two particular cases fell within a range convenient to include on a single graph. From such a plot, the 50% reaction point (threshold) can be read directly. The standard error of the threshold is obtained by the method of Miller and Tainter (1944). The tabulated results presented below are derived from data on 69 individual beetles tested in three different runs in

the liquid phase, and from data on 150 individuals tested in three different runs in the case of the gas thresholds. This difference in numbers results from the different sized populations convenient to test in the different sized reaction chambers of the two types of apparatus. An analysis of the variance between reactions of the three different groups of beetles tested at each concentration of stimulus showed no significant difference between the reactions of the three groups in either phase and the data were therefore combined and treated as though derived from a single homogeneous population.

When beetles were to be operated upon, they were anesthetized with carbon dioxide and lifted onto clay blocks, having depressions into which the animals fit with their ventral sides uppermost. A broad rubber band around the block covered the posterior halves of the beetles and held them in position during the operations.

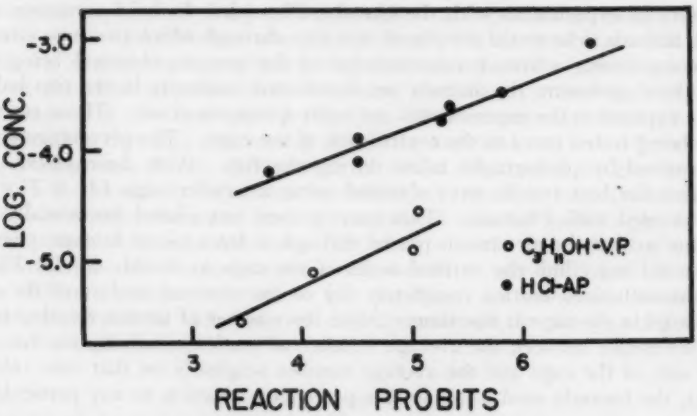



FIGURE 1. Relationship between percentage reaction and quantity of stimulating chemicals. Data in this plot obtained from experiments on unoperated beetles exposed to 1-pentanol in the vapor phase and HCl in the aqueous phase.

Watchmaker's forceps and iridectomy scissors were used to grasp and remove whatever portions of the head appendages were desired. The point at which the appendages were severed when they were to be completely removed is indicated in Figure 2. Other operations were performed as noted blow. The effects of the anesthetic wore off within five minutes and the operated beetles seemed to have no difficulty swimming or flying.

To restrict the investigation to a manageable size, the chemicals used were restricted to 1-pentanol, HCl, and NaCl. The first two compounds can be conveniently worked with in the vapor phase and enough data on their thermodynamic and other properties exist to permit comparisons of possible mechanisms of action in the two phases. The latter two compounds represent classic modalities (acid and salt) of taste stimuli which were the source of the conflicting data cited in the introduction. It should be noted that although it would have been desirable to include a sugar among the aqueous phase stimulating compounds, *Laccophilus* does

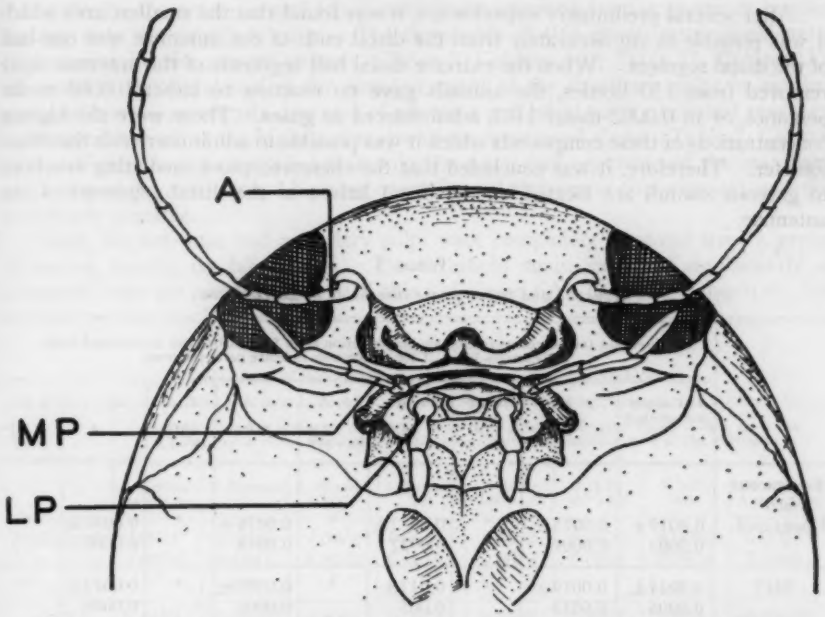



FIGURE 2. Ventral view of *Laccophilus* head, showing antennae and palpi. The pointers indicate the positions near the base of each appendage where they were severed if they were to be entirely removed.

not react to aqueous solutions of sucrose when tested by this method with the highest concentration it is possible to use in the apparatus and still maintain normal rates of flow (approximately one molar sucrose).

## RESULTS

1. *The identity of receptors mediating reactions in the gas and liquid phases.* (Threshold data are summarized and compared in Tables I and II.)

Beetles were exposed to 1-pentanol and HCl in both phases and thresholds determined for the normal (unoperated) animals. As noted above, data from three groups are treated as one homogeneous unit to derive each threshold value. The results are given in Table I, Columns 1A and 1B. Then the antennae were completely removed from another series of animals and a similar run made in both phases (Table I, Columns 8A and 8B). Since it appeared that a complete loss of sensitivity to the gaseous stimuli, within the range of concentrations it was possible to test with the present apparatus, was suffered by beetles with antennae removed, it was not necessary to go on to other mouthparts in attempting to localize the receptors mediating reactions to gases, and a more precise localization of the gas phase receptors on the antennae was sought.

After several preliminary experiments, it was found that the smallest area which it was possible to cut accurately from the distal ends of the antennae was one-half of the distal segment. When the extreme distal half segments of the antennae were removed from 150 beetles, the animals gave no reaction to either 0.0003 molar pentanol, or to 0.0002 molar HCl, administered as gases. These were the highest concentrations of these compounds which it was possible to administer with the olfactometer. Therefore, it was concluded that the chemoreceptors mediating reactions to gaseous stimuli are located on the distal halves of the distal segments of the antennae.

TABLE I

*Aqueous and vapor phase thresholds of Laccophilus*

| | Threshold (molar concentration evoking reaction from 50% of beetles) ± standard error. Appendages listed at top of columns indicate parts removed. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1. Control (unoperated) | 2. MP LP | 3. MP LP ½S−A | 4. A LP | 5. A LP ½S−MP | 6. A MP | 7. A MP ½S−LP | 8. A | 9. A MP LP |
| *A. Aqueous phase* | | | | | | | | | |
| 1-pentanol | 0.0019± 0.0003 | 0.0015± 0.0007 | * | 0.0082± 0.0022 | * | 0.0076± 0.0010 | * | 0.0050± 0.0007 | * |
| HCl | 0.0014± 0.0006 | 0.0019± 0.0010 | * | 0.012± 0.005 | * | 0.0090± 0.0008 | * | 0.0073± 0.0006 | * |
| NaCl | 0.17± 0.03 | 0.18± 0.08 | * | 0.78± 0.13 | * | 0.73± 0.19 | * | 0.48± 0.09 | * |
| *B. Vapor phase* | | | | | | | | | |
| 1-pentanol | 0.00015± 0.00008 | 0.00027± 0.00011 | — | — | — | — | — | * | — |
| HCl | 0.00012± 0.00003 | 0.00014± 0.00005 | — | — | — | — | — | * | — |

Abbreviations and symbols used: A, antennae; MP, maxillary palpi; LP, labial palpi; S, distal segments; *, no reaction to highest concentration tested.

To see if the same area was involved in mediating reactions to these chemicals in aqueous solution, the distal halves of the distal segments of the antennae were removed from 69 other beetles, previously deprived of palpi—which, it will be shown, bear other chemoreceptors—and these beetles exposed to pentanol and HCl (Table I, Column 3A). These beetles no longer reacted, even to stimuli as concentrated as 0.1 molar pentanol and 0.5 molar HCl, which are, respectively, fifty and three hundred times as concentrated as would be necessary to stimulate 50% of the unoperated animals.

At first it was not known where the other receptors mediating reactions to chemicals in the liquid phase were located. On the basis of Schaller's experiments it was expected that they would be located on the palpi and in the mouth cavity.

Accordingly, the antennae and labial palpi were removed from a group of beetles, leaving only the maxillary palpi. The thresholds of this group were significantly higher than for the normal animals (Table I, Column 4A; Table II, Column 3). Removal of the distal half of the distal segment of the maxillary palpi in another group of beetles, previously deprived of antennae and labial palpi, abolished reactions to chemical stimuli at the highest concentrations mentioned above (Table I, Column 5A). Hence, it was concluded that only the distal half-segments of the maxillary palpi bear chemoreceptors. Further operative subdivision of this segment was not technically possible.

Next, the antennae and maxillary palpi were completely removed from a group of beetles, leaving the labial palpi. The threshold again increased significantly as compared with the normal animals (Table I, Column 6A; Table II, Column 4), but

TABLE II

*Comparison of thresholds of receptor groups in Laccophilus*

| Compound | Receptor areas or operative conditions being compared. The upper figure of each set is the difference in molar threshold, and the lower figure the standard error of the difference | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1. Normal; minus A | 2. Normal; A | 3. Normal; MP | 4. Normal; LP | 5. MP; MP+LP | 6. LP; MP+LP | 7. LP; MP | 8. Normal; A (gas phase) |
| 1-pentanol | 0.0031<br>0.00095<br>* | 0.0005<br>0.0011 | 0.0063<br>0.0022<br>* | 0.0057<br>0.00105<br>* | 0.0032<br>0.0023 | 0.0026<br>0.0013 | 0.0006<br>0.0024 | 0.00012<br>0.0080 |
| HCl | 0.0059<br>0.00085<br>* | 0.0005<br>0.0012 | 0.0096<br>0.005<br>* | 0.0076<br>0.0010<br>* | 0.0057<br>0.0050 | 0.0017<br>0.0010 | 0.003<br>0.0051 | 0.00002<br>0.000044 |
| NaCl | 0.31<br>0.095<br>* | 0.01<br>0.085 | 0.61<br>0.13<br>* | 0.56<br>0.19<br>* | 0.30<br>0.16 | 0.25<br>0.21 | 0.05<br>0.23 | —<br>— |

An asterisk indicates a significant difference (more than twice the standard error of the difference) between thresholds. Abbreviations as in Table I. Based on tests in aqueous phase except as otherwise noted.

did not differ significantly from the threshold of the receptors on the maxillary palpi alone (Table II, Column 7), or from the threshold of the combined maxillary and labial palpi (Table II, Column 6). Removal of the distal segments of the labial palpi from a group of beetles previously deprived of their antennae and maxillary palpi abolished all reaction to the highest concentrations of chemical stimuli tested (Table I, Column 7A). In the case of the labial palpi, too, the chemoreceptors must be on the distal segment, although the small size of this segment precluded any further localization by ablation techniques.

The comparisons presented in Table II show that the only significant changes in threshold occur when the antennae of the beetles are removed, regardless of the phase in which the stimulus is administered. The high concentrations to which the "refractory" beetles were exposed without obtaining any reaction make it extremely improbable that the beetles ever employ chemoreceptors within the buccal cavity or elsewhere on the body in these experiments.

*2. The specificity of the chemoreceptor groups*

From the data already presented, it appears unlikely that any one appendage bears chemoreceptors sensitive to only one particular modality of stimulus, as suggested by Ritter (1936). As additional evidence on this point, the aqueous phase thresholds of the antennae and palpi to NaCl were determined and the results are included at appropriate places in Table I. It is clear that the receptor area most sensitive to HCl and pentanol (distal tip of the antennae) is also the most sensitive to NaCl, and that the palpi are again of approximately equal sensitivity, as was the case with the alcohol and acid. When the tips of the antennae were removed from animals previously deprived of palpi, the beetles gave no reaction to 1.5 molar NaCl, the highest concentration tested. The question of specialization of various receptors within a single group or area is considered in the discussion.

*3. Structure of the chemoreceptors*

On the basis of evidence thus far presented, it is clear that the chemoreceptors must be located within the distal halves of the distal segments of the antennae, maxillary palpi, and labial palpi. The greater sensitivity of the antennae in both phases suggests that some difference might exist either in the morphological characteristics of the receptors on the antennae and palpi or that the number of receptors might be greater on the antennae. A morphological study of the antennae and palpi was made to discover if any such specializations of structure or number of receptors were apparent. Some of the appendages removed from the operated animals were used for this purpose, and some parts were removed especially for this purpose so that their orientation on the slides could be checked.

The antennae and palpi were placed in a drop of Clarite on a microscope slide and the slide placed in a vacuum chamber. A fifteen-pound vacuum was sustained in the chamber for twenty minutes. This removed any air bubbles formed within the cut end of the material to be observed. After cover slips were in place, the whole-mounts were observed with magnifications up to 1300 ×. Camera lucida drawings of the distal segment, or portions thereof, from the antennae and palpi are shown in Figure 3. All the parts are drawn as they would be seen from the ventral side when the orientation corresponds to that shown in Figure 2. Since, in the living beetles, these appendages are usually waving around vigorously, this manner of orientation may be clearer than trying to orient their surfaces with reference only to the dorsal and ventral aspects of the beetle's body. The magnification scale for *A* and *B* is given in the upper left of the figure and the scale for *C* in the upper right. The entire distal segments of the palpi are shown, and the remainder of the distal antennal segment lacked other receptors.

The key to the presumed sense organs, following the classification of Snodgrass (1935), is as follows: 1-sensilla chaetica; 2-sensilla basiconica; 3-sensilla coeloconica; 4-sensilla placoidea. The sensilla basiconica of the palpi are the same in appearance as those on the antennae, although it was necessary to represent them diagrammatically on this drawing because of their numbers, which are at least several hundred on each palpus. Snodgrass describes the sensilla basiconica as probable chemoreceptors, but little experimental evidence on this point is available. Perhaps the most pertinent evidence is that of Roth and Willis (1951) which strongly indicates that the sensilla basiconica are hygroreceptors in *Tribolium*. It will be

noted that the arrangement of receptors in *Laccophilus* makes it practically certain that the sensilla basiconica are the chemoreceptors with which the present experiments are concerned. The only other morphologically distinguishable receptors found in all three of the receptor-bearing areas are the sensilla chaetica and other evidence, as noted below, makes their importance in chemoreception very highly improbable. For example, the sensilla chaetica were found on the other flagellar segments of the antennae and were morphologically and numerically identical on the other segments to those on the distal segment, as well as occupying similar positions on the segments. The same is true for the coeloconic sensillum which is also

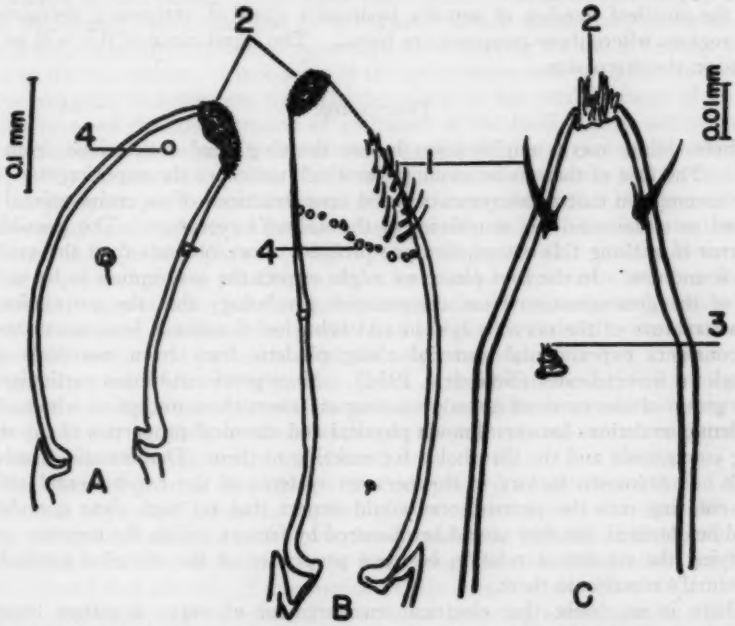



FIGURE 3. Receptors on antennae and palpi. A—distal segment of labial palp; B—distal segment of maxillary palp; C—a portion of the distal segment of an antenna. Key to receptor types and additional explanation in text.

present on other flagellar segments of the antennae. Hence, if receptors other than the sensilla basiconica were chemoreceptors on the antennae, it would be necessary to assume that similar morphological types of receptors situated in corresponding positions on other antennal receptors were functioning in some entirely different manner. This hypothesis is rejected in favor of the simpler alternative—*i.e.*, that the sensilla basiconica of the antennae are chemoreceptors, and that the other receptor types which are also present elsewhere are not chemoreceptors. The evidence with regard to the palpi is more equivocal. Sensilla chaetica are more abundant (by a factor of four) on the proximal segment of the labial palpi than on the distal segment, and are at least as abundant on the two basal segments of the maxillary

palpi as on the distal segment. Hence, it is unlikely that they are chemoreceptors. The sensilla placoidea are also possible chemoreceptors on the palpi. However, since only one of these organs is present on the distal segment of the labial palpi, it seems unlikely that it alone would be responsible for all the sensitivity of the labial palpi to chemicals. In the case of the maxillary palpi, where the placoid organs circle the distal segment, the placoid organs may be chemoreceptors.

It may be concluded that the sensilla basiconica are the chemoreceptors on the antennae and the receptors chiefly responsible for the sensitivity of this species to gaseous and liquid stimuli. There is also evidence that sensilla basiconica are chemoreceptors on the palpi. It is surprising that the most sensitive region contains the smallest number of sensilla basiconica (tips of antennae) compared to other regions where these receptors are found. The significance of this will be considered in the discussion.

## Discussion

Three points merit amplification before drawing final conclusions from this study. The first of these is an assumption which underlies the experiments. This is the assumption that measurements based upon reactions of an entire animal may be used to understand the sensitivity of the animal's receptors. The possibilities for error in making this assumption are probably more obvious than the evidence of its soundness. In the first place, we might expect the assumption to be valid in view of the generalization from comparative psychology that the correlation between structure of the nervous system and behavior of animals becomes clearer as one considers experimental material along phyletic lines from mammals down through the invertebrates (Schneirla, 1952). More pertinent in this particular case is the group of observations already existing on insect chemoreception which shows excellent correlations between known physical and chemical properties of the stimulating compounds and the thresholds for reaction to them (Dethier and Chadwick, 1948). If unknown factors in the nervous systems of the experimental animals were entering into the picture, one would expect that no such clear correlations would be obtained, for they would be obscured by factors within the nervous system modifying the consistent relation between properties of the stimulus applied and the animal's reaction to them.

There is no doubt that electrical measurement of nerve impulses from the chemoreceptors would be the method of choice for use on invertebrates as well as vertebrates, but thus far this has been either technically impossible (Jahn and Wulff, 1950) or limited to detection of narcotic effects which are not specific effects of the stimulating chemicals on chemoreceptors (Hodgson and Roys, unpublished). It is hoped that eventually it will also be technically possible to limit chemoreception studies to a single receptor cell, but in the meantime much of interest may come to light while working toward that end.

In view of the finding that a very small group of antennal receptors mediates reactions to different modalities of chemicals in either gas or liquid phases, it seems logical to next inquire why the receptors on the palpi were not observed to mediate reactions in both phases, since they were sensitive to all three test compounds in the aqueous phase. This discrepancy is resolved by the observation that the thresholds of the palpi are significantly higher than those of the antennae and are, in fact, higher than the concentrations of chemicals which it is possible to accurately ad-

minister with the present olfactometer, which reaches its upper limit of concentrations at about one doubling concentration higher than the threshold of these beetles to either pentanol or HCl vapors. Although it is theoretically quite conceivable that some reactions might be obtained by exposing the receptors on the palpi to very concentrated gases, it seems quite certain that in nature the high thresholds of the palpi would prohibit their participation in mediating reactions of the animals to gaseous stimuli.

The difference in threshold obtained when the antennal receptors were tested in gas and liquid phases is, however, of some interest with regard to possible limiting mechanisms in each phase. If the same factor limits the effectiveness of one chemical in stimulating chemoreceptors in two phases, then it should be possible to bring the differences in thresholds into agreement by correcting for the difference in that factor in the two phases. Since some of the information now available on chemoreception suggests that the process is characterized by the establishment of an equilibrium between the concentration of stimulant at the basic site of action and the concentration of stimulant in the phase external to the organism (Dethier and Yost, 1952), it was thought that the limiting factor determining the thresholds in the two phases might be establishment of such an equilibrium. If this were true, the thermodynamic activity of the stimulant, suitably defined, should have the same numerical value in both phases (Ferguson and Pirie, 1948). On the basis of the present experiments, it is possible to make four such comparisons between thermodynamic activities of stimuli at threshold concentrations in aqueous and vapor phases. These values were calculated for pentanol and HCl at threshold concentrations for unoperated beetles, and for beetles with the palpi removed, according to the methods of Ferguson and Pirie (1948), and Brink and Posternak (1948). Since the thermodynamic activities at threshold were not even of the same order of magnitude when data from similarly treated beetles in the two phases were compared, this approach will not be elaborated upon here.

The essential point is that either chemoreception does not depend upon the establishment of an equilibrium in the two phases or else some behavioral difference in the testing methods used in the two phases masks the identity of the fundamental mechanisms. In this connection it is interesting to note that Dethier and Yost (1952) found that alcohols of intermediate chain length when stimulating blowflies as gases obeyed the law of equal effect at equal thermodynamic activity; however, this principle did not hold when the tarsal receptors of the blowfly are stimulated by alcohols, although here, too, the behavioral criteria might be involved in the discrepancy.

Finally, the bearing that these findings have on the concepts of olfaction, gustation, and sensory modalities should be mentioned. It must be conceded that classifying stimuli as odor-substances or taste-substances is simply making a difficult problem more obscure and is a practice which should be abandoned. In view of the present results, the advisability of making a distinction between olfactory and gustatory receptors on the basis of the physical state of the stimulus to which they are sensitive could be similarly misleading, since a very restricted group of receptors of the same morphological type can mediate reactions to both gaseous and liquid stimuli. It is remarkable that the smallest number of sensilla basiconica is on the region most sensitive to chemicals and a much larger number on the palpi which are relatively insensitive. This indicates that the sensitivity of the antennal

receptors is the result of some specialization of the receptor cells themselves rather than simply the result of a great many receptors summing nerve impulses as is sometimes advanced by way of an explanation for the greater sensitivity of olfaction as compared with gustation (Moncrieff, 1944).

There is no evidence in these data for the existence of topographically separated receptors sensitive only to particular modalities of stimuli as was concluded by Ritter (1936). It is possible that the apparent specificity of the receptors on the labial palpi for acids resulted from having the concentrations of salt and non-acid stimuli below threshold, while the acid happened to be above the threshold of the receptors on the labial palpi. This particular point is more relevant to understanding chemoreception in insects than to concepts about the chemical senses in general, since it merely indicates that the sense organs of insects have not evolved the same types of specializations as those found in mammals. Of course it cannot be assumed that different sensilla within a particular group necessarily have identical physiological functions and thresholds, any more than it could be assumed, for example, that different sensilla with cuticle of similar thickness have the same permeability properties (Richards, 1952). Such a condition would be, however, quite different from the grouping of specialized receptors suggested by Ritter (1936). This leaves the problem of the existence of modalities among chemicals stimulating for insects, and especially the basis of their discrimination by the animal, in a very unsatisfactory state. If different classes of chemicals do not activate different receptors, how are they discriminated as classes of stimuli? One wonders how many such classes of compounds might be found in a systematic experimental survey not organized around the classic modalities of taste stimuli for humans. Much additional evidence on this point is needed, but the suggestion by Frings (1946) that the modalities represent only points in a continuous spectrum of taste sensations related to some surface-active property of the chemicals might offer a less anthropomorphic approach for experimentation than some of those used in the past.

## Summary

1. Quantitatively controlled stimuli were administered to populations of an amphibious beetle, *Laccophilus maculosus* Germar, to determine whether the same chemoreceptors are sensitive to gaseous and liquid stimuli, and to discover qualitative or quantitative specializations in the function of different receptor groups.

2. Sensilla basiconica on the tips of the antennae are the principal chemoreceptors for both gaseous and liquid stimuli. The lower threshold of antennal receptors, relative to receptors elsewhere on the animals, is not due to a larger number of sensilla on the antennae and indicates an inherent specialization of the receptors themselves.

3. Morphological and experimental evidence strongly indicates that the sensilla basiconica on the tips of the maxillary and labial palpi also function as chemoreceptors for stimuli in solution, although their thresholds are higher than those of antennal receptors. The concentration of gases could not be raised to a level adequate for stimulation of receptors on the palpi and they must play little or no part in mediating reactions of the animals in air.

4. HCl, 1-pentanol, and NaCl all stimulated receptor areas on the tips of the antennae and palpi, and no evidence was found for specialization of any morphologically or topographically distinguishable receptor groups sensitive to only a particular

modality of stimulus. Chemoreceptors were not found on parts of the body other than the antennae and palpi.

5. The findings are discussed with reference to possible identity of fundamental mechanisms limiting the effectiveness of chemical stimuli in the two physical states, and general concepts of olfaction and gustation.

## LITERATURE CITED


BAUER, L., 1938. Geschmäcksphysiologische Untersuchungen an Wasserkäfern. *Zeitschr. vergl. Physiol.*, **26**: 107–120.

BLISS, C. I., 1938. The determination of the dosage-mortality curve from small numbers. *Quart. J. Pharm. and Pharmacol.*, **11**: 192–216.

BRINK, F., AND J. M. POSTERNAK, 1948. Thermodynamic analysis of the relative effectiveness of narcotics. *J. Cell. Comp. Physiol.*, **32**: 211–234.

COLE, W. H., AND J. B. ALLISON, 1930. Chemical stimulation by alcohols in the barnacle, the frog, and planaria. *J. Gen. Physiol.*, **14**: 71–86.

DETHIER, V. G., AND L. E. CHADWICK, 1948. Chemoreception in insects. *Physiol. Rev.*, **28**: 220–254.

DETHIER, V. G., AND M. T. YOST, 1952. Olfactory stimulation of blowflies by homologous alcohols. *J. Gen. Physiol.*, **35**: 823–839.

FERGUSON, J., AND H. PIRIE, 1948. The toxicity of vapours to the grain weevil. *Ann. Appl. Biol.*, **35**: 532–550.

FRINGS, H., 1946. Gustatory thresholds for sucrose and electrolytes for the cockroach, *Periplanata americana* (Linn). *J. Exp. Zool.*, **102**: 23–50.

VON FRISCH, K., 1924. Sinnesphysiologie der Wassertiere. Verh. deutsch. Zool. Gesellsch., pp. 21–42.

HODGSON, E. S., 1951. Reaction thresholds of an aquatic beetle, *Laccophilus maculosus* Germ., to salts and alcohols. *Physiol. Zool.*, **24**: 131–140.

JAHN, T. L., AND V. J. WULFF, 1950. Chemoreception. In: C. L. Prosser (ed.), Comparative animal physiology. W. B. Saunders, Philadelphia.

LEASURE, J. K., 1939. The mucus sheet on the respiratory mucous membrane. *Trans. Amer. Acad. Opthalmol. Otol.*, **44**: 341–347.

MATTHES, E., 1924. Das Geruchsvermögen von *Triton* beim Aufenthalt unter Wasser. *Zeitschr. vergl. Physiol.*, **1**: 57–83.

MATTHES, E., 1927. Der Einfluss des Mediumwechsels auf das Geruchsvermögen von *Triton*. *Zeitschr. vergl. Physiol.*, **5**: 83–166.

MILLER, L. C., AND M. L. TAINTER, 1944. Estimation of the $ED_{50}$ and its error by means of logarithmic-probit graph paper. *Proc. Soc. Exp. Biol. and Med.*, **57**: 261–264.

MONCRIEFF, R. W., 1944. The chemical senses. John Wiley & Co., New York.

MURPHY, W. E., 1931. Anaphylactic changes in the nasal mucosa. *Arch. Otolaryngol.*, **13**: 842–845.

NAGEL, W., 1894. Ergebnisse vergleichend-physiologischer und anatomischer Untersuchungen über den Geruchs- und Geschmäcksinn und ihre Organe. *Biol. Zentrbl.*, **14**: 543–555.

PARKER, G. H., AND R. E. SHELDON, 1913. The sense of smell in fishes. U. S. Bur. Fisheries Document No. 775.

RICHARDS, A. G., 1952. Studies on arthropod cuticle. VIII. The antennal cuticle of honeybees, with particular reference to the sense plates. *Biol. Bull.*, **103**: 201–225.

RITTER, E., 1936. Untersuchungen über chemischen Sinn beim schwarzen Kolbenwasserkafer *Hydrous piceus*. *Zeitschr. vergl. Physiol.*, **23**: 543–570.

ROTH, L. M., AND E. R. WILLIS, 1951. Hygroreceptors in Coleoptera. *J. Exp. Zool.*, **117**: 451–487.

SCHALLER, A., 1926. Sinnesphysiologische und psychologische Untersuchungen an Wasserkäfern und Fischen. *Zeitschr. vergl. Physiol.*, **4**: 370–464.

SCHNEIRLA, T. C., 1952. A consideration of some conceptual trends in comparative psychology. *Psychol. Bull.*, **49**: 559–597.

SNODGRASS, R. E., 1935. Principles of insect morphology. McGraw-Hill, New York.

WALKER, T. J., AND A. D. HASLER, 1949. Detection and discrimination of odors of aquatic plants by the bluntnose minnow (*Hyborhynchus notatus*). *Physiol. Zool.*, **22**: 45–63.

# THE PERMEABILITY OF THE ERYTHROCYTE-LIKE CELLS OF PHASCOLOSOMA GOULDI [1]

WARNER E. LOVE

*The Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa., and The Marine Biological Laboratory, Woods Hole, Mass.*

The coelomic fluid of the annelid worm *Phascolosoma gouldi* contains cells of unknown function which at least superficially resemble vertebrate erythrocytes (Cuénot, 1891; Dawson, 1933). Like the latter, they have two special properties which permit the use of Jacobs' hemolysis method (1934) to determine their permeability constants. First, they lose their red pigment, hemerythrin, on swelling osmotically to a certain critical volume; the accompanying optical changes are as striking as those seen in the hemolysis of vertebrate erythrocytes. Second, their discoidal shape enables one to assume that the cell surface area does not change significantly during moderate osmotic swelling.

This paper reports the application of Jacobs' method to Phascolosoma "erythrocytes." Measurements have been made of (1) normal cell volume, (2) "hemolytic" volume, (3) cell surface area, and (4) "hemolysis" times in water and various solutions isosmotic with sea water. From these data, permeability constants have been calculated and compared with those of certain representative cells for which quantitative data exist.

## Material and Methods

To make stock suspensions of Phascolosoma erythrocytes, coelomic fluid from ten to twenty worms was diluted to about 100 cc. with filtered sea water, which Chapeau (1928) has reported to be isotonic. Filtration through a loose plug of absorbent cotton removed the large eggs and the clot which usually formed. Repeated gentle centrifugation served to separate the erythrocytes from the much smaller sperm cells which remained suspended. The packed cells from the final centrifugation were used as the stock suspension.

Normal cell volume was determined from 8 hematocrits combined with 5 cell counts of the same suspension. The average cell volume was 1050 $\mu^3$.

It was obviously impossible to measure accurately the hemolytic volume during hemolysis. Instead, it was estimated from microscopic measurements of the diameters of intact spherical cells remaining in the dilute sea water which previously had been found to produce 50% hemolysis. The mean diameter of fifty such cells was 14.5 $\mu$ with a standard deviation of 1.8 $\mu$; thus the average hemolytic volume was 1610 $\mu^3$, and the average surface area was 660 $\mu^2$, assuming constancy during swelling. The swollen cell volume, calculated for the 60% sea water used above by the van't Hoff law from the normal cell volume, exceeds the measured hemolytic volume by an amount corresponding to a non-solvent space of 20% of the cell.

[1] These studies were supported by a grant from The National Science Foundation.

This "dead-space" is higher than that of 7–14% for Arbacia eggs found by McCutcheon *et al.* (1931) and lower than that of about 30% reported by Ponder (1948) for mammalian erythrocytes. Although it is recognized that there may conceivably be a selective effect of this procedure for estimating hemolytic volume, the method has been used because of convenience. Its use is supported by the fact that the non-osmotic volume so estimated is in reasonable agreement with the high hemerythrin content of these cells (Florkin, 1933).

The non-electrolyte solutions were made one molar, which is osmotically roughly equivalent to Woods Hole sea water, and were lightly buffered to approximately the pH of sea water (by addition of 5% by volume of 0.33 *M* $Na_2HPO_4$ which had been brought to pH 7.8 with concentrated HCl). As a result, the pH remained essentially constant during hemolysis of added cells. One-half molar solutions of ammonium salts of fatty acids were prepared by titrating a small quantity of the proper undiluted fatty acid with *M/2* $NH_4OH$ to a pH such that equal amounts of ammonia and fatty acid were present in the solutions (see, in this connection, Stewart, 1934). To measure a hemolysis time in one of these solutions, 0.05 cc. of the stock suspension of cells was mixed with 10 cc. of the solution; the time of 50% hemolysis was determined by the "slit" method of Jacobs *et al.* (1949). Control experiments showed that none of the substances discussed below were hemolytic *per se*.

## Results and Discussion

The observed hemolysis times are listed in the second column of Table I. In the third column are listed the corresponding permeability constants, calculated by the method of Jacobs (1934) from hemolysis times, hemolytic volume, and normal cell volume and surface area. For comparison the permeability constants of unfertilized Arbacia eggs (Jacobs and Stewart, 1932), those of cells of *Chara ceratophylla* (Collander and Bärlund, 1933) and those of beef erythrocytes (Jacobs, 1952) are also given. To fill out the table, the permeability constants of the beef erythrocyte for amides were determined by Jacobs' hemolysis method. They are in good agreement with the experiments of Höber and Ørskov (1933). Following Collander and Bärlund (1933), for solutes P is the number of mols crossing one cm.$^2$ of membrane in one hour with a concentration difference of one mol per cc., while $P_w$ is the cc. of water crossing one cm.$^2$ of membrane with an osmotic pressure difference of one atmosphere.

Two general qualitative principles have emerged from the past studies of permeability: (1) small molecules diffuse into living cells faster than larger ones, (2) lipoid-soluble molecules diffuse into living cells faster than those which are not lipophilic (Höber, 1945). Phascolosoma erythrocytes follow the above rules rather well, as is indicated by their permeability constants in Table I. For example, permeability to alkyl amides increases with increasing hydrocarbon chain length. This probably results from a stronger influence of lipoid solubility than of molecular volume on diffusibility of the molecules. Probably for the same reason esterification of glycerol with acetic acid increases the rate of entrance. On the other hand, in the series of glycols where molecular volume increases without much change in lipoid solubility, the expected decrease in the ease of penetration is seen. Further agreement with the properties of other cells is shown by the increased permeability

to 1,3 propandiol versus glycerol, as well as higher permeability to 1,2 propandiol than to its 1,3 isomer.

The results in Table I show that Phascolosoma coelomic cells resemble the other nucleated cells and that they do not possess the special high permeability to both water and urea of beef erythrocytes. In this respect the cells here studied do resemble the erythrocytes of the elasmobranchs, amphibia and reptiles, although only qualitative comparisons can be made with the data of Jacobs *et al.* (1950). In

TABLE I

*Permeability constants of selected cells for various substances*

| Substance | Time in seconds for 50% hemolysis of Phascolosoma "erythrocytes" at 25° C. | $P \times 10^4$ (cm. $\times$ hr.$^{-1}$) | | | |
|---|---|---|---|---|---|
| | Solute 1 Molar | Phascolosoma | Arbacia eggs | Chara leaf cells | Beef red cells |
| Formamide | 158 | 59 | | 770† | 1760 |
| Acetamide | 124 | 95 | 345‡ | 530† | 1700 |
| Propionamide | 73 | 374 | 850‡ | 1300† | 685 |
| Butyramide | 53 | 2460 | 2200‡ | 1700† | 980 |
| Ethylene Glycol | 185 | 44 | 240* | 430† | 58* |
| Diethylene Glycol | 281 | 21 | 150* | | 27* |
| Triethylene Glycol | 637 | 7.1 | | | 12* |
| Tetraethylene Glycol | 1573 | 2.5 | | | |
| Urea | 552 | 9.6 | | 40† | 6500* |
| Thiourea | 423 | 12.1 | | 77† | 7* |
| | more than | less than | | | |
| Glycerol | 14,400 | 0.025 | 3‡ | 7† | 0.7* |
| Monacetin | 1137 | 3.7 | | 160† | 41* |
| Diacetin | 248 | 26.8 | | 800† | 375* |
| 1,3 Propandiol | 186 | 44 | 260* | | 36* |
| 1,2 Propandiol | 145 | 69 | 460* | 870† | 118* |
| | | $P_w$ (cm.$^4$ $\times$ hr.$^{-1}$ $\times$ mol.$^{-1}$) | | | |
| Water | 49.2 | 72 | 22§ (exosmosis)<br>18‖ (endosmosis) | | 400* |

* Jacobs (1952); † Collander and Bärlund (1933); ‡ Jacobs and Stewart (1932) § Jacobs (1932); ‖ Lucké, Hartline and McCutcheon (1931).

addition, in the beef erythrocyte the effect of lipoid solubility on ascending the homologous series of amides is not apparent from the first member of the series as it is with Arbacia eggs and Phascolosoma erythrocytes.

Experiments with electrolytes throw further light on this question of the general classification of Phascolosoma erythrocytes with respect to their permeability. Their lysis is quite rapid in $M/2$ ammonium acetate, but does not occur in four hours in either $M/2$ ammonium chloride or $M/2$ sodium acetate. The cells there-

fore appear to be permeable to both ammonia and acetic acid and evidently much less permeable to either sodium ions or chloride ions (Jacobs, 1927). In this respect, therefore, they resemble typical plant and animal cells rather than the vertebrate erythrocyte, in which a special free permeability to anions facilitates the transport of carbon dioxide.

Permeability constants calculated from the hemolysis times in isosmotic solutions of ammonium salts of fatty acids do not have the same significance as those already mentioned because of the complex manner in which these salts enter cells (Jacobs, 1927). However, the hemolysis times shown in Table II, like the results obtained with other cells (Jacobs, 1927; Bouillenne, 1930; Stewart, 1934), indicate that increasing lipoid solubility of the fatty acid goes hand in hand with an increase in the rate of entrance of the salt. It should be noted that in the series: acetate, propionate, butyrate, and valerate, the lysis times are entirely comparable because the pK values of the four acids in question are nearly the same.

TABLE II

*50% hemolysis times in M/2 solutions of ammonium salts of fatty acids*

| Ammonium salt | Time in seconds for 50% hemolysis of Phascolosoma erythrocytes at 25° C. |
|---|---|
| Formate | 1,145 |
| Acetate | 388 |
| Propionate | 158 |
| Butyrate | 93 |
| Valerate | 52 |

The hemerythrin-containing cells of Phascolosoma coelomic fluid resemble the erythrocytes of the lower vertebrates morphologically. Moreover, they contain a high concentration of a red pigment which combines loosely and reversibly with oxygen (Cuénot, 1891), and which they lose by a process analogous to hemolysis of vertebrate erythrocytes. Yet in most instances the present results show that substances enter the Phascolosoma cells more nearly the way they enter Chara cells and Arbacia eggs; in particular, Phascolosoma erythrocytes lack the special free permeability to urea, water, and anions characteristic of the beef erythrocyte.

The author wishes to express his gratitude to Prof. M. H. Jacobs for his help during the course of the work, and to Dr. T. F. Anderson for his aid in preparing the manuscript.

## SUMMARY

By virtue of two properties, lysis on reaching a critical volume, and a discoidal shape, the hemerythrin-containing corpuscles of the coelomic fluid of *Phascolosoma gouldi* have proven to be excellent material for the quantitative estimation of permeability constants. The constants evaluated by the method of Jacobs from times of lysis show a general similarity between these cells and unfertilized Arbacia eggs in respect to permeability to water and a series of non-electrolytes. The Phascolosoma cells also resemble Arbacia eggs in their low permeability to both cations and anions.

## LITERATURE CITED


BOUILLENNE, R., 1930. Pénétration des sels ammoniacaux dans les cellules végétales. *C. R. Soc. de Biol.*, **103**: 50–52.

CHAPEAU, M., 1928. Recherches sur la respiration des hématies du siponcle. *Bull. Sta. Biol. D'Arachon*, **25**: 157–187.

COLLANDER, R., AND H. BÄRLUND, 1933. Permeabilitätsstudien an *Chara ceratophylla*. *Acta Botan. Fennica*, **11**: 1–114.

CUÉNOT, L., 1891. Études sur le sang les glandes lymphatiques dans la serie animale 2ª Partie: Invertébrés. *Arch. de Zool. Exper. et Gen. 2ª Ser.*, **9**: 593–670.

DAWSON, A. B., 1933. Supravital studies on the colored corpuscles of several marine invertebrates. *Biol. Bull.*, **64**: 233–242.

FLORKIN, M., 1933. Le pouvoir oxyphorique du liquide coelomique et des hématies de *Sipunculus nudus*. *C. R. Soc. de Biol.*, **112**: 705–706.

HÖBER, R., 1945. Physical chemistry of cells and tissues. The Blakiston Co., Philadelphia.

HÖBER, R., AND S. L. ØRSKOV, 1933. Untersuchungen über die Permiergeschwindigkeit von Anelektrolyten bei den roten Blutkörperchen verschiedener Tierarten. *Arch. ges. Physiol.*, **231**: 599–615.

JACOBS, M. H., 1927. The exchange of material between the erythrocyte and its surroundings. *Harvey Lectures*, **22**: 146–164.

JACOBS, M. H., 1932. The simultaneous measurement of cell permeability to water and to dissolved substances. *J. Cell. Comp. Physiol.*, **2**: 427–444.

JACOBS, M. H., 1934. The quantitative measurement of the permeability of the erythrocyte to water and solutes by the hemolysis method. *J. Cell. Comp. Physiol.*, **4**: 161–182.

JACOBS, M. H., 1952. Modern trends in physiology. (E. S. G. Barron, ed.) Academic Press, New York.

JACOBS, M. H., H. N. GLASSMAN AND A. K. PARPART, 1950. Hemolysis and zoological relationship. Comparative studies with four penetrating non-electrolytes. *J. Exp. Zool.*, **113**: 277–300.

JACOBS, M. H., AND D. R. STEWART, 1932. A simple method for the quantitative measurement of cell permeability. *J. Cell. Comp. Physiol.*, **1**: 71–82.

JACOBS, M. H., D. R. STEWART, W. J. BROWN AND L. J. KIMMELMANN, 1949. An improved method for the detection of osmotic abnormalities of erythrocytes. *Amer. J. Med. Sci.*, **217**: 47–52.

LUCKÉ, B. H., K. HARTLINE AND M. MCCUTCHEON, 1931. Further studies on the kinetics of osmosis in living cells. *J. Gen. Physiol.*, **14**: 405–419.

MCCUTCHEON, M., B. LUCKÉ AND H. K. HARTLINE, 1931. The osmotic properties of living cells (eggs of *Arbacia punctulata*). *J. Gen. Physiol.*, **14**: 393–403.

PONDER, E., 1948. Hemolysis and related phenomena. Grune and Stratton, New York.

STEWART, D. R., 1934. The permeability of the *Arbacia* egg to ammonium salts. *Biol. Bull.*, **60**: 171–178.

# NUCLEO-CYTOPLASMIC INTERACTION DURING CONJUGATION IN TETRAHYMENA[1]

DAVID L. NANNEY[2]

*Department of Zoology, University of Michigan, Ann Arbor, Michigan*

Studies on the ciliated Protozoa, particularly *Paramecium aurelia* (Sonneborn, 1950b, 1951), have provided much information on the roles of the nucleus and the cytoplasm in cellular differentiation. One important observation in these studies (Sonneborn, 1947) is that the cytoplasm may control the kind of macronucleus developed in a cell; specifically, the cytoplasm may determine whether a new macronucleus will differentiate so as to control one or the other of the possible mating types. It has been suggested (Nanney, 1953) that the cytoplasm in these cells has been determined by the kind of macronucleus previously occupying the cell. This does not detract from the importance of the cytoplasm in cellular heredity, but emphasizes the importance of nucleo-cytoplasmic *interactions*.

Related to this problem of what determines the *kind* of macronucleus to develop is the problem of what determines *whether* a particular nucleus will differentiate into a macronucleus. In many ciliates the fertilization nucleus produced at nuclear reorganization divides twice to produce four presumably identical nuclei: two of these differentiate as macronuclei and two as micronuclei. Long ago Maupas (1889) suggested that this difference in the development of nuclei was due to localized differences in the cytoplasm surrounding the nuclei at a critical time in their development. Maupas based this suggestion on observations made on a group of ciliates, including particularly *Colpidium, Leucophrys* and *Glaucoma*. He observed that in these organisms the fertilization nucleus divided twice and that the spindles for the second post-zygotic division were oriented in such a fashion that two of the four division products in each cell were placed at the extreme anterior end of the cell and two were placed at the extreme posterior end of the cell. Those which were placed at the anterior end of the cell were observed to enlarge and become the new macronuclei while those at the posterior end remained small and became the new micronuclei. Here was a clear visible correlation between the location of a nucleus in the cytoplasm and its subsequent development. Although this correlation strongly suggested cytoplasmic control of nuclear development, other interpretations were possible and were not excluded.

A number of observations similar to those of Maupas have been made on a variety of organisms since Maupas' time and recently Sonneborn (1951) has directed attention to the nuclear events at conjugation in *Paramecium* where several additional instances of apparent cytoplasmic control of nuclear activity are

[1] This work was supported by a Faculty Research Grant, No. 270, from the Horace H. Rackham School of Graduate Studies of the University of Michigan.

[2] The author wishes to express his appreciation to Prof. A. M. Elliott, who provided the strains of *Tetrahymena* used in the present study. He is also grateful to Prof. T. M. Sonneborn and Dr. Ruth V. Dippell for many helpful criticisms in the preparation of the maunscript.

found. The present study is presented as an extension of his observations to another organism, with certain experimental data bearing on his conclusions.

## Materials and Methods

Strains of *Tetrahymena* were recently obtained (Elliott and Nanney, 1952) which permitted further analysis of some of these problems. These strains, designated as the AA strains, undergo conjugation regularly. The entire process has been analyzed both to gain insight into the factors controlling nuclear development and to provide a firm basis for subsequent genetic studies. The eight strains studied were all collected in the vicinity of Ann Arbor, Michigan and in each of them any isolated cell will give rise to a culture in which conjugation (selfing) occurs. However, no conjugants have been found which give rise to viable progeny. More recently (Elliott and Gruchy, 1952), other strains of *Tetrahymena* were collected near Woods Hole, Massachusetts, and in these strains—designated as the WH strains—Elliott has demonstrated that mating types exist and that viable progeny are produced. The details of conjugation in the WH strains resemble closely those in the AA strains with the exception of a few modifications related to the occurrence of mating types and a high frequency of spontaneous anomalies in the cytogenetic processes. These differences and their significance will be discussed in a later paper.

The selfing strains are designated as AA 1–8, and have been assigned to the genus *Tetrahymena* by Corliss and Furgason (in Elliott and Nanney, 1952). They show certain differences from the strains of *T. pyriformis* (=*gelii*) extensively studied and are, hence, described simply as *T. sp.* One of the principal differences between these strains and the long-maintained laboratory cultures of *T. pyriformis* lies in the fact that the latter are lacking in micronuclei. *T. sp.* has, as a rule, a single micronucleus, but occasional cells with as many as four micronuclei have been observed.

Most of these strains, perhaps all, can be grown on a defined medium (Elliott and Nanney, 1952), but in the present study all the cells were maintained in bacterized cultures. The medium was prepared by boiling 1½ grams of Cerophyl in a liter of distilled water, filtering and autoclaving. The day before the medium was to be used, it was inoculated with *Aerobacter aerogenes*. The general culture methods followed closely those described for *Paramecium* by Sonneborn (1950a).

Conjugation occurs regularly in all the stocks soon after the nutrient in the medium is exhausted. It has not been possible even after many serial isolations to derive cultures differing in mating type; any isolation gives rise to a clone within which nearly 100% conjugation can occur. No evidence was found for autogamy or any other process of nuclear reorganization which might account for diversities within a culture; hence, it seems reasonable to assume that the cells which conjugate are genetically alike. The question of whether the cells are of different mating types will be discussed in a later paper, but at the present time no evidence is available for any differences between the cells that conjugate.

Under all conditions thus far tried, the exconjugants die—usually without separating. The cultures are perpetuated from the individuals which have failed to conjugate. Attempts to obtain viable conjugants by growing cells from different sources in the same container and isolating conjugants have proved unsuccessful.

Since conjugation occurs within a single culture, it is difficult to control its initiation. Pairs are formed over a period of several hours and samples removed at any one time contain pairs in many stages of conjugation. The sequence of stages must, therefore, be inferred rather than directly demonstrated. Similarly the length of time necessary for the completion of the various stages cannot be determined readily.

In preparation for cytological studies, pairs were killed and fixed in hot Schaudinn's solution. Various staining techniques were used: these include the borax-carmine method of Dippell (in Sonneborn, 1950a), Dippell and Chao's (in Sonneborn, 1950a) modification of the De Lamater stain and the Giemsa method described by Preer (1950).

The figures representing the sequence of stages are camera lucida drawings of stained pairs. No attempt has been made to simulate the structural details of the nuclei or to indicate the precise number or size of the chromosomes. The chromosomes are small, numerous and difficult to count or draw, though this should be possible eventually. Characteristic changes may be noted in the staining properties of the chromosomes and these will be described later.

## Descriptive

### 1. *The normal pattern of nuclear behavior*

The normal pattern of nuclear behavior during conjugation closely resembles that reported by Maupas (1889) for *Leucophrys patula*. Recent systematic revisions (Furgason, 1940; Corliss, 1952) indicate that *Leucophrys patula* is more correctly termed *Tetrahymena patula*, a species closely related to *T. sp.;* hence, this similarity in nuclear behavior is not surprising. In spite of the similarity in the cytogenetic details reported by Maupas and those reported below, it appears advisable to present briefly the normal sequence of events as a basis for comparison with the anomalies to be presented subsequently.

Conjugating cells attach at their oral surfaces (near the anterior ends of the cells) with a "face to face" orientation. Preliminary clumping reactions and nonspecific attachments have not been observed, but it is possible that these occur. At the time the cells first become attached, the single micronucleus is found near the macronucleus but may be anterior, posterior or lateral to it. Shortly after the initiation of conjugation the micronucleus moves into a region just anterior to the macronucleus (Fig. 1A), enlarges and begins to elongate into the typical "crescent" stage of the first pre-zygotic division (Fig. 1B). This crescent shortens in the later stages of this division (Fig. 1C) and the chromosomes become clearly visible. The first division is completed and the two daughter nuclei enter immediately into the second pre-zygotic division (Fig. 1D) still anterior to the macronucleus. During this division the chromosomes are much less prominent than during the previous division. Extrapolating from information on other ciliates whose genetics are well known (see Sonneborn, 1947), it appears probable that these first two divisions are the meiotic divisions and that the resulting four nuclei are haploid.

At the end of the second pre-zygotic division the four nuclei in each cell continue to migrate anteriorly until one of the nuclei comes in contact with the membrane between the cells and appears to attach to it. More specifically, the nuclei attach on the right side of the cell. The attachment of nuclei on opposite sides of the contact

surfaces is usually, but not always, synchronous. Following the attachment of one of the nuclei, the remaining nuclei in the cell begin to move posteriorly (Fig. 1E *et seq.*), eventually to disintegrate. These "relic" nuclei may persist for a variable length of time, but have never been observed to undergo any further divisions.

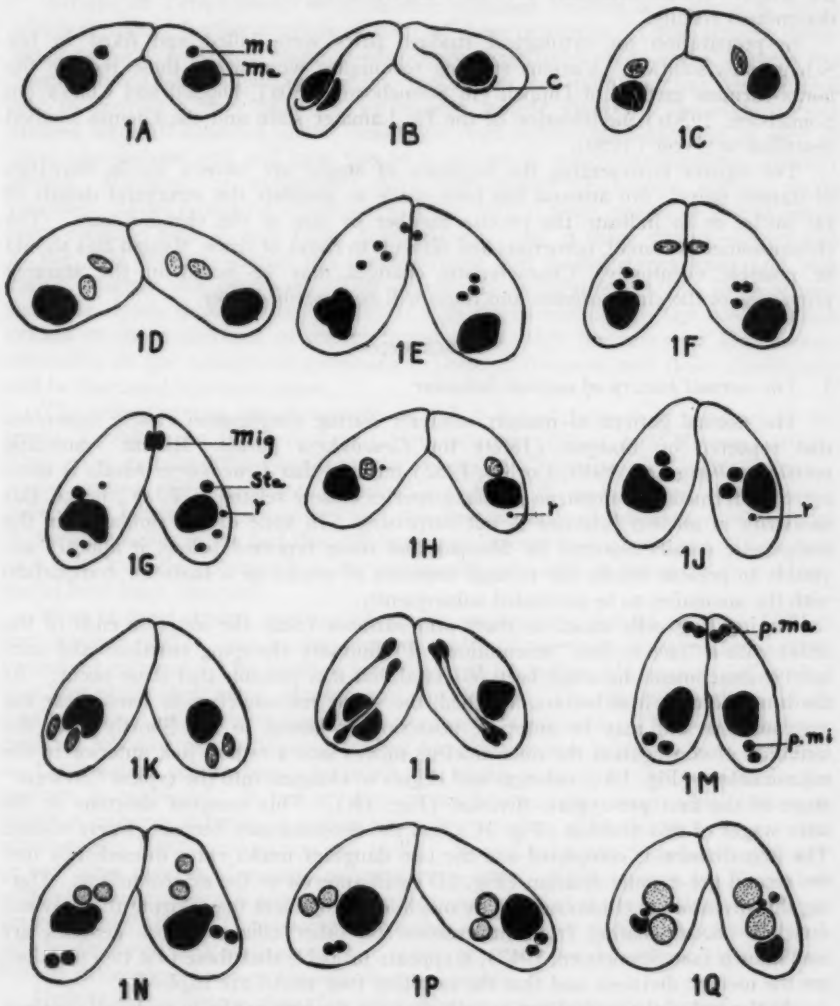



FIGURE 1. The normal sequence of nuclear changes at conjugation in *Tetrahymena sp.*; *mi* = micronucleus; *ma* = macronucleus; *c* = crescent; *r* = relic nuclei; *mig* = migratory nucleus; *sta* = stationary nucleus; *s* = syncaryon; *p. ma* = presumptive macronucleus; *p. mi* = presumptive micronucleus. See explanation in text.

The nucleus situated at the membrane enlarges and undergoes a third prezygotic division while still attached (Fig. 1F). Since the previous divisions are probably the meiotic divisions, this may be assumed to be an equational division. One of the nuclei produced by this division (the migratory nucleus) remains at its original position; the other nucleus (the stationary nucleus) moves to a region just anterior to the macronucleus (Fig. 1G) and may be distinguished from the relic nuclei by its larger size and more spherical shape. By this time these other haploid nuclei are often pycnotic and in some instances have already disintegrated.

The migratory nucleus is observed to flatten considerably (Fig. 1G) and then to round up and protrude slightly into the other cell. Eventually the migratory nuclei from the two pair members are exchanged. The fertilization nucleus is formed by the fusion, just anterior to the macronucleus, of the migratory nucleus from one cell with the stationary nucleus from the other cell. This fertilization nucleus immediately prepares to divide (Fig. 1H). Since the fertilization nucleus is formed by the union of presumably identical nuclei in the two members of a pair, it is necessary that the two exconjugants of a single pair will be alike in their genetic constitution.

Immediately following fertilization the syncaryon undergoes its first post-zygotic division while still anterior to the macronucleus. The two large conspicuous daughter nuclei (Fig. 1J) migrate posteriorly and the second and last post-zygotic nuclear division is initiated posterior to the macronucleus with the spindles oriented longitudinally in the cell (Fig. 1K). These spindles elongate until they extend nearly the entire length of the cell (Fig. 1L), and at the end of this division two nuclei are left at the extreme anterior end of the cell and two at the extreme posterior end of the cell (Fig. 1M). Those at the anterior end, the presumptive macronuclear anlagen, begin to move toward the posterior end, to enlarge and stain less intensely than before (Fig. 1N and 1P). The nuclei at the posterior end, on the other hand, remain small and become the new micronuclei.

At about this time the original macronucleus shows the first evidence of change. It loses its irregular outline, becomes spherical and deeply staining (Fig. 1P). Eventually it becomes smaller and is lost (Figs. 1Q–2B), although not invariably at the same time in both conjugants.

The pair members usually do not separate, though they may be forcibly separated during the latest stages and a few pairs separate spontaneously. Often the conjugating cells coalesce (Fig. 2C), become spherical and vacuolated, and finally lyse. The conjugants which separate likewise do not survive.

### 2. *Conjugation involving three cells*

Maupas (1889) reported having seen triple formations many times in *Leucophrys patula*, as well as in other ciliates, but gave no further information on either the mode of attachment or on the cytogenetic details. Triples are regularly seen in the AA cultures and may involve a small percentage of the observed conjugants. In all cases thus far observed the triples are formed by the symmetrical union of cells at the oral surfaces. No attachments at other points have been observed. In the WH *Tetrahymena* strains a different kind of triple has been found. These triples are due to the simultaneous union of two single animals to the two oral surfaces of a "double" animal. Such triples are not to be confused with the

tripolar triples reported here. A third type of triple is known in many ciliates and occurs when a third cell becomes attached in any of a variety of positions to one member of a conjugating pair. In *Paramecium bursaria*, Chen (1946) has shown that this third mate undergoes autogamy and neither receives a pronucleus from nor contributes a pronucleus to either of the other cells. Weisz (1950) reports triple formations in *Blepharisma*, some of which appear to be similar to those studied by Chen, but others of which may be of the tripolar sort described below

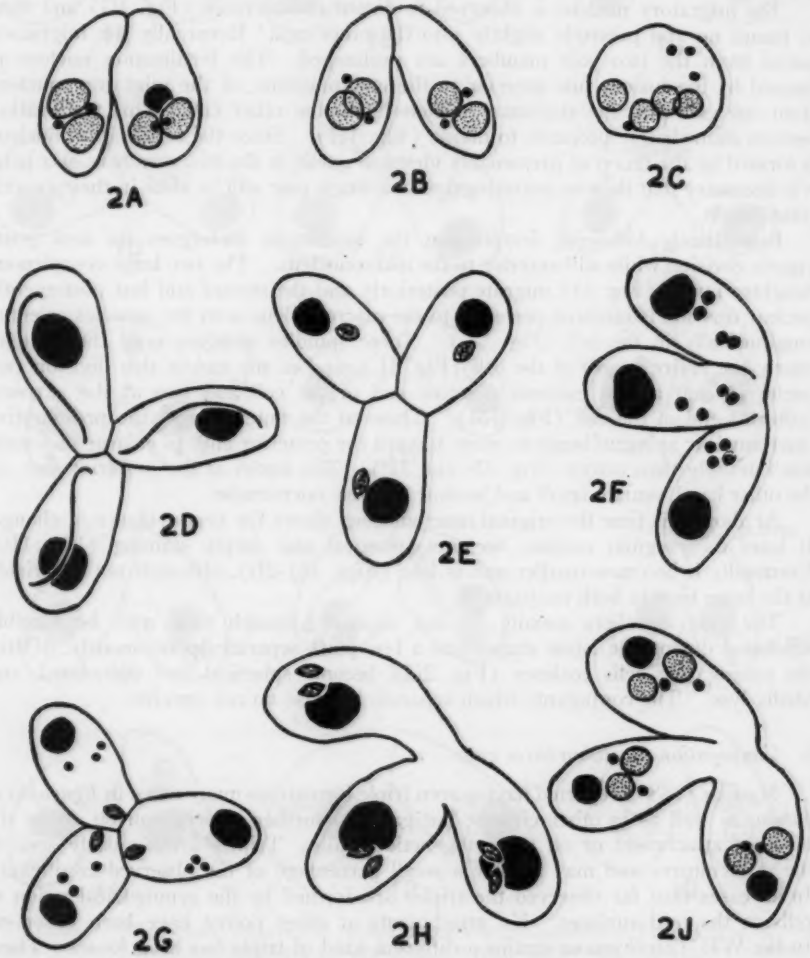



FIGURE 2. 2A–2C, terminal stages in normal conjugation. 2D–2J, conjugation in triples. See explanation in text.

for *Tetrahymena*. Since nuclear studies on the *Blepharisma* strains were not conclusive, this interpretation must remain tentative.

The pattern of nuclear behavior in the AA triples is precisely identical with that in pairs; all three cells appear to undergo a normal reorganization with the normal synchrony. Figure 2D shows a typical crescent stage; Figure 2E shows a later stage in the first pre-zygotic division. Figure 2F shows the four nuclei in each cell produced by the second pre-zygotic division. Figure 2G shows the spindles for the third pre-zygotic division. It will be noted that the dividing nuclei are all attached on what appears to be the left side of the cell (actually the right side, judging from the nuclear orientation in pairs where right and left are readily determined). It appears likely that in this case the migratory nucleus passes through the membrane to which it is attached and, hence, that each cell contributes a migratory nucleus to one cell and receives a migratory nucleus from the other cell. This is, therefore, in all probability a true tripolar fertilization and should yield different genetic results than normal conjugants. Specifically, tripolar fertilization could yield three genetically diverse cells after conjugation under some circumstances. Figure 2H shows the spindles for the second post-zygotic nuclear division and Figure 2J illustrates the stage after the new macronuclei have differentiated, but before the old macronucleus has completely disappeared.

### 3. *Conjugation in cells with multiple micronuclei*

Within mass cultures of several of the stocks occasional cells have been observed with multiple micronuclei. In one isolation line nearly all the cells showed two micronuclei at the time they were first stained. Subsequently the frequency of bi-micronucleate cells decreased and the culture returned to the uni-micronucleate condition. During the period when the culture possessed many bi-micronucleate cells, pairs were stained and studied.

Multiple micronuclei appear in no way to affect the behavior of the individual nuclei or to alter the consequences of conjugation. All the micronuclei originally present undergo the first and second pre-zygotic divisions. Figure 3A shows a pair in which both conjugants have two micronuclei and in which all the micronuclei are in the crescent stage. Figure 3B shows a pair in which one cell has one micronucleus and the other has two. Figures 3C–3E show the later stages in the first pre-zygotic division in conjugants with a variety of nuclear constitutions. Figure 3F shows the beginning of the second pre-zygotic division in a pair which originally consisted of a uni-micronucleate and a bi-micronucleate member. The chromatin in this stage appears as a faintly staining network, strikingly different from the chromatin in Figures 3C–3E, in which distinct chromosomes are readily seen. Figure 3G shows a pair similar to that in 3F, but after the second pre-zygotic division; eight nuclei are seen in one cell and four in the other. Figure 3H shows a pair beginning the third pre-zygotic nuclear division; the one dividing nucleus and the seven relic nuclei in each cell indicate that both cells were originally bi-micronucleate. It is observed that only one micronucleus ever undergoes the third pre-zygotic division, regardless of whether a cell contains four, eight or twelve (when the cell was originally tri-micronucleate). Following fertilization and the disintegration of the relic nuclei, no differences can be ascertained between cells

which were originally multi-micronucleate and those that were originally uni-micronucleate.

The number of micronuclei present in a cell appears to have no influence on either the probability of mating or on the kinds of matings observed. The frequency of multi-micronucleate cells in conjugation is not significantly different from the frequency of such cells in the same culture which are not conjugating at

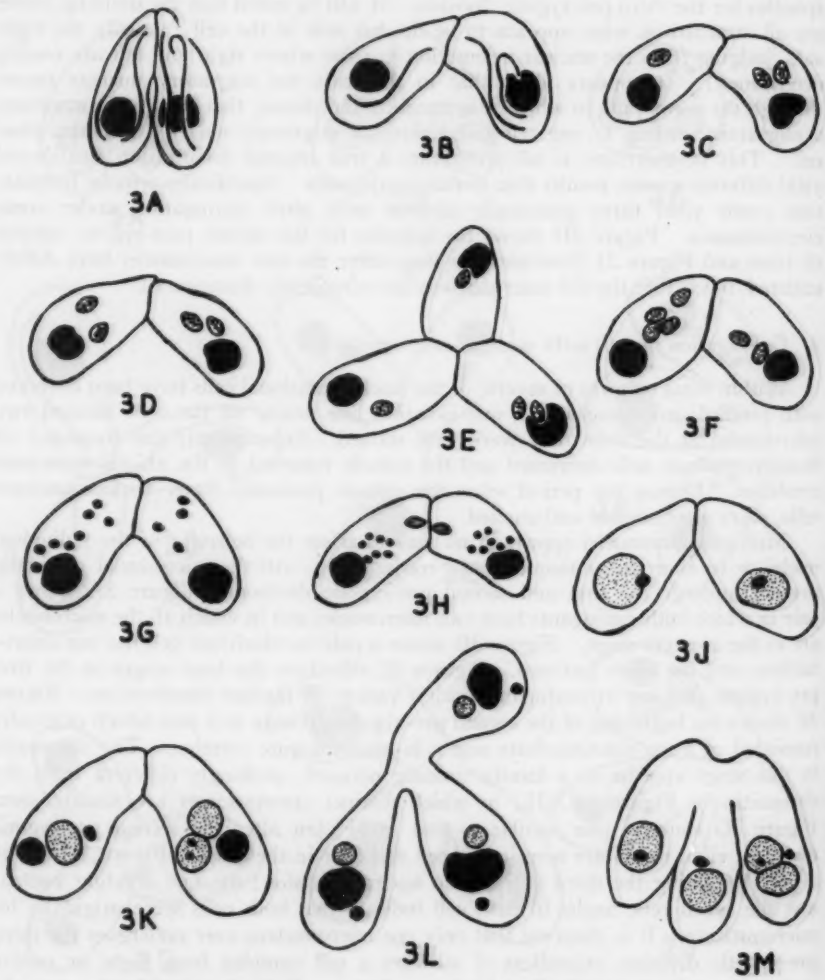



FIGURE 3. 3A–3H, conjugation in cells with multiple micronuclei. 3J–3M, a spontaneous anomaly. See explanation in text.

a particular time. The distribution of pair types (uni-micronucleate × uni-micronucleate, uni-micronucleate × bi-micronucleate, etc.) is not significantly different from the distribution expected by chance alone.

4. *A spontaneous anomaly*

Only one spontaneously occurring anomaly has been observed in the AA cultures, and this occurred in about 7% of the conjugants (45 of 608 on one slide) in a single stock (AA-1) the first time conjugation was observed; it has not been encountered since. Unfortunately, the culture in which the anomaly was found was in the later stages of conjugation, and it was not possible to determine the manner in which the abnormality developed. This anomaly is characterized by the presence at the end of conjugation of only one new macronucleus and only one new micronucleus instead of the usual two of each kind (Fig. 3J–3M). These new nuclei, both macro- and micronuclei, are clearly larger than those in normal cells. Rough measurements show a difference in volume of a factor of two compared with the nuclei in comparable stages of normal cells.

An examination of prepared slides was undertaken to determine whether pair members tended to resemble each other in respect to the number of new nuclei produced. On a particular slide 270 pairs were found, in which both conjugants were normal; 23 pairs were found with one abnormal member and 11 pairs were observed to have two abnormal members. On simple probability considerations the expected classes are 260, 42 and 2. It seems probable, therefore, that pair members tend to be alike. The significance of this observation is not clear.

## Experimental

The fact that differences in the behavior of different nuclei present in the cell at the same time are correlated with the regular localization of the nuclei in specific cytoplasmic regions is sufficient to suggest that nuclear behavior is to some extent controlled by local differences in the cytoplasm. This fact alone, however, may not be considered critical evidence for such a cytoplasmic role. It is conceivable that the nuclei are self-determined to behave as they do regardless of where they are located. If, however, nuclei could be transferred from one cytoplasmic locality to another and if this relocation could be demonstrated to result in altered nuclear behavior or nuclear differentiation, the hypothesis of cytoplasmic control could be considered firmly established.

The simplest method for relocating nuclei in the cytoplasm appeared to be centrifuging. Cultures in conjugation were centrifuged in an International Clinical Centrifuge at full speed (about 5000 g) for various lengths of time; they were allowed to recover for from one to 24 hours and were then fixed and stained. Some cultures were centrifuged for ten minutes; other cultures were centrifuged for ten minutes, allowed to recover for fifteen minutes and were again centrifuged. In some instances three periods of centrifugation were used.

Cultures stained immediately after centrifugation showed that the nuclei were indeed relocated in the cytoplasm, and slides prepared at various intervals after the cells had recovered showed that alterations in nuclear behavior had been accomplished. These alterations may be described as follows.

1. *Simple mate-to-mate transfer*

The commonest abnormality observed was the transfer of part of the nuclear equipment from one cell into its mate. Observations suggest that this transfer may occur during any stage of conjugation. Figure 4A shows a pair in which the entire micronuclear material of one cell was transferred to the other cell at some stage prior to fertilization and probably prior to the attachment of a nucleus to the membranes separating the cells. One cell contains no micronuclei; the other contains seven

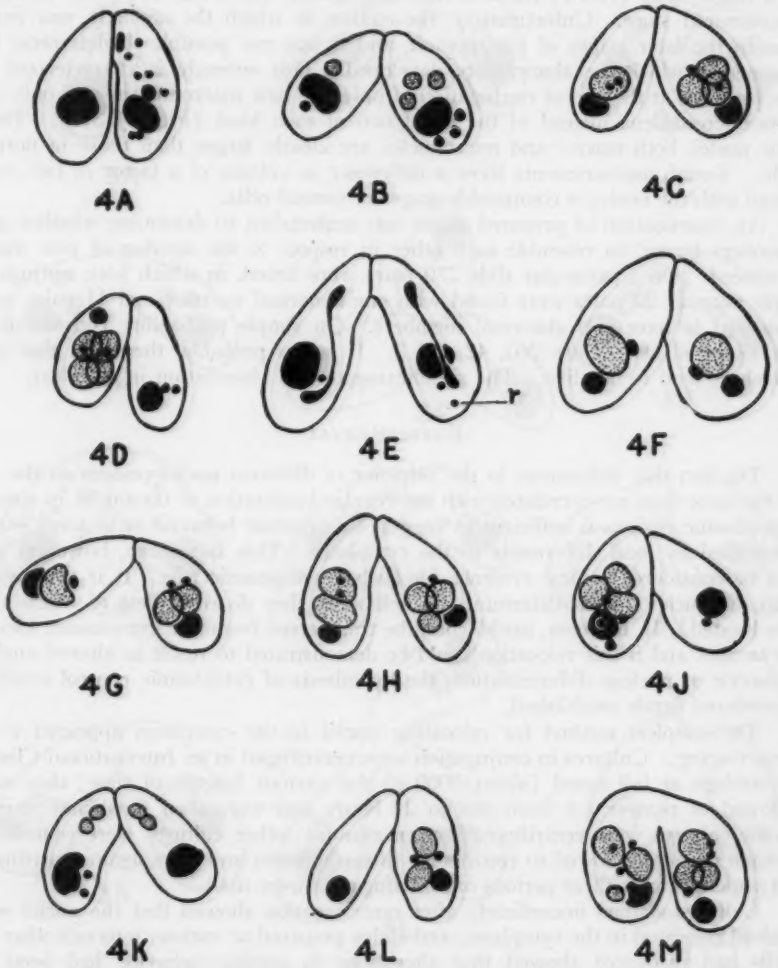



FIGURE 4. Abnormalities in conjugation induced by centrifugation. See explanation in text.

relic nuclei, a migratory nucleus and a stationary nucleus. This could be interpreted as conjugation between an amicronucleate and a bi-micronucleate cell were it not for the fact that *no* amicronucleate and *no* bi-micronucleate cells were observed elsewhere in the culture. Figure 4B is most readily interpreted as due to a mate-to-mate transfer after fertilization and presumably just after the first post-zygotic division. If this interpretation is correct, each nucleus continued to develop in its normal manner to give rise to a macronucleus and a micronucleus. Either of these cases could also be interpreted on the basis of a dual effect of the centrifugation—an elimination of nuclei from one cell and an induced extra division of nuclei in the other cell, but the usual compensation for the loss of nuclei in one cell by the addition of nuclei to its mate makes the mate-to-mate transfer interpretation more likely. In other cases where there is no compensation, the interpretation of nuclear loss or induced extra divisions may be more seriously entertained.

Mate-to-mate transfers at later stages, *i.e.*, after the differentiation of micronuclei and macronuclei, are more common. Figure 4C shows a pair in which one of the macronuclei from one cell was transferred to the other cell and Figure 4D shows a pair in which both new macronuclei were transferred from one cell into its mate. These observations show no evidence for cytoplasmic control of nuclear processes, but demonstrate clearly that relocation of nuclei in the cytoplasm does occur during centrifugation. Other, but rarer, types of abnormalities are more illuminating. Some of these are associated with mate-to-mate transfer and others are presumably due merely to alterations in the positions of the nuclei within a single cell.

2. *Alterations in the number of nuclei*

The most common abnormality observed—next to simple mate-to-mate transfer—was essentially like the spontaneous abnormality discussed above. In some cells only two new nuclei were formed in contrast to the usual four, and the new nuclei were larger than normal nuclei in comparable stages. Figure 4E shows a pair in which both members show a spindle like the typical spindle for the second post-zygotic division, but each pair shows only one such spindle. Figure 4F shows a similar pair at a later stage and Figure 4G shows a pair in which the anomaly occurred in only one cell of a pair. These abnormalities can be explained on two simple assumptions: (1) the cytoplasm at the posterior end of the cell determines that a nucleus under its influence will undergo a "final" division with a longitudinally oriented spindle, and (2) the centrifugation resulted in a fertilization nucleus being placed under this influence before it had the opportunity to undergo a normal first post-zygotic division. If this interpretation is correct, the spontaneous abnormality may be similarly explained on the basis of some unspecified environmental influence that causes the fertilization nucleus to arrive at the posterior end prematurely. An alternative interpretation would hold that the figures shown represent conjugants from which some of the nuclei have been removed by centrifugation. If this is true, the experimentally induced aberrations are not necessarily related to the spontaneous aberrations where nuclear loss seems unlikely.

3. *Alterations in nuclear development*

Another kind of aberration noted involves the change of a presumptive micronucleus into a macronucleus or of a presumptive macronucleus into a micronucleus.

Since it is not possible to follow any particular pair through conjugation, such alterations can be detected only as cells containing abnormal relative numbers of micronuclei and macronuclei. Figure 4H shows a pair which appears to satisfy expectations. One pair-member is normal, *i.e.*, it contains two micronuclei and two macronuclei. The other pair-member shows two well developed macronuclei and one typical micronucleus. The other new nucleus, presumably derived from the other presumptive micronucleus, is much larger, less deeply staining, and appears to be in the process of developing into a macronucleus. This may be interpreted as a nucleus whose developmental route was altered at a fairly late stage. Whether it would develop into a fully formed new macronucleus is of course not known. Figure 4J shows the reciprocal transformation, in this case combined with mate-to-mate transfer. One cell contains only a single micronucleus; the other contains four micronuclei and three new macronuclei. The pair as a whole has, therefore, produced five micronuclei and three macronuclei. It appears reasonable to assume that one of the presumptive macronuclei has given rise to a micronucleus. Since aberrations of this sort have been observed in the AA strains only when the cells are centrifuged, and since centrifugation certainly relocates the nuclei in the cytoplasm, it seems reasonable to assume that the relocation itself results in an alteration in nuclear development.

4. *Other alterations*

Other alterations in nuclear development have also been observed, but these are much rarer and an insufficient number of each kind has been observed to warrant extended discussion. A few of these will be illustrated. Figure 4K shows a pair in which the nuclei in one cell are normal, while the other cell contains only two nuclei—both developing as macronuclei. This can be interpreted by assuming two effects of the treatment—an alteration in nuclear number followed by the transformation of a presumptive micronucleus into a macronucleus. Since this pair had been centrifuged three times during conjugation, this interpretation does not seem improbable.

Figure 4L shows a pair consisting of one apparently normal cell and one with no new nuclei. This may be interpreted as due to the transfer of the fertilization nucleus from one cell into its mate followed by a single division of each of the nuclei. It could also be explained by a mate-to-mate transfer at an earlier stage be present, additional evidence for alteration in nuclear development is seen in the nuclei from one cell.

Figure 4M is more difficult to interpret since the total number of new nuclei in each cell is six rather than the usual four. This could be due to an extra division of one of the products of the first post-zygotic divisions in each cell. This result might also be found if this was originally a triple, the extra nuclei being derived from a third cell before it was lost. Regardless of how the multiple nuclei came to be present, additional evidence for alteration in nuclear development is seen in the small partially developed macronucleus in the left member.

## Discussion

Sonneborn (1951) pointed out several instances where cytoplasmic control of nuclear behavior was indicated in the cytogenetic processes of *Paramecium*.

Particularly he suggested that cytoplasmic locations were determinative in the following instances: 1) the survival or disintegration of haploid nuclei following meiosis; 2) the differences in the behavior of the migratory and stationary nuclei; 3) the differentiation of micronuclei and macronuclei. His conclusions may be extended with little modification to *Tetrahymena* and additional instances of cytoplasmic control may also be suggested.

In proceeding to a discussion of the factors involved in nuclear behavior at conjugation in *Tetrahymena*, it is necessary to point out the general features of this behavior. These may be discussed under the following headings: nuclear migration, nuclear division and nuclear differentiation.

### 1. *Nuclear migration*

The various stages of nuclear reorganization are characterized by events occurring in specificially localized regions of the cytoplasm. The first question raised is whether the movements resulting in the specific localizations are autonomous, or whether the cytoplasm controls these movements to some extent. This question may not be answered with certainty but certain considerations favor the latter solution. The location of the nuclei may be understood in terms of two migrations, the first a migration toward the anterior end of the cell (toward the contact membranes) and the second a migration toward the posterior end of the cell (away from the contact membranes). Before conjugation the micronuclei occupy positions near the macronucleus, but may be either anterior or posterior to it; the first and second pre-zygotic division figures are always anterior to the macronucleus. After meiosis the nuclei continue to move anteriorly until one of the haploid nuclei attaches to the membrane between the cells. This terminates the anterior migration; all subsequent movements, except those resulting from nuclear displacement during nuclear division, are from the anterior end of the cell to the posterior end. After one nucleus attaches, the relic nuclei begin to move posteriorly and, if they do not disintegrate first, come to lie at the extreme posterior end of the cell. The fertilization nucleus also migrates posteriorly from its position in front of the old macronucleus, and the new macronuclei move posteriorly from the position where they were placed by the elongated spindles of the second post-zygotic division.

The anterior migration is initiated at the time the cells come in contact at their oral surfaces; it is terminated at the time a nucleus attaches at these same surfaces. There is thus circumstantial evidence that events occurring at the contact surface determine nuclear migrations. Several possibilities are available in regard to the nature of such events, but in the absence of further evidence, speculation appears unprofitable.

### 2. *Nuclear divisions*

The nuclear divisions are as follows: two meiotic divisions, a pre-zygotic equational division of one of the haploid nuclei and usually two post-zygotic divisions. The meiotic divisions, like the nuclear migrations, are initiated following the attachment of the cells. The pre-zygotic equational division is clearly related to the cytoplasmic disposition of the nuclei since it occurs only in the nuclei attached to the contact membranes. The specificity of this cytoplasmic location is shown by the

fact that attachment is always on the right side of the cell. The nature of these contact membranes, the manner in which they adhere to one another, the way they initiate the process of conjugation and control nuclear movements (if indeed they do), the manner in which they assure the transfer of presumably identical nuclei in opposite directions are all problems of importance in understanding the complex phenomena of conjugation. These problems require much further study.

The post-zygotic nuclear divisions are also controlled to some extent by the cytoplasmic conditions, but these conditions are not obviously related to the contact membranes. The first post-zygotic division occurs anterior to the old macronucleus; the second occurs posterior to the old macronucleus. Evidence has been presented which suggests that if the first post-zygotic division occurs posterior to the macronucleus, this is the final division, giving rise to a macronucleus and a micronucleus. Since the division occurring anterior to the macronucleus under normal circumstances shows a spindle oriented transversely in the cell, whereas all divisions occurring posterior to the macronucleus show spindles oriented longitudinally in the cell, the influence of this cytoplasm may lie in or be associated with its control of spindle orientation.

### 3. *Nuclear differentiation*

Several types of nuclear differentiation are noted during the conjugation process. The first differentiation is that of enlargement of the micronuclei prior to meiosis. Like the nuclear migrations and meiosis, this enlargement is directly or indirectly related to events occurring at the contact surfaces. The second differentiation is that occurring in the haploid nucleus attached to the membranes. The unattached nuclei disintegrate without dividing; the attached nucleus divides and both its daughter nuclei persist. Particularly it is to be noted that the daughter nucleus free in the same cytoplasm with the disintegrating nuclei does not distintegrate. It must, therefore, be different from them, though it is extremely unlikely that this difference is genetic; both types of nuclei are presumably haploid and must reasonably be expected to be alike in genetic material in many instances. The size difference between the stationary nucleus and the relic nuclei is further evidence for some kind of differentiation. Sonneborn (personal communication) suggests, on the basis of observations on *Paramecium*, that a transient cytoplasmic condition initiates the degeneration of free nuclei at a particular time, even though complete disintegration is not observed until later. According to this view, the stationary nucleus is released into the cytoplasm when the cytoplasm is no longer capable of initiating degeneration.

The differences in the behavior of the migratory and the stationary nuclei may also be ascribed to cytoplasmic relations, but it is possible that the differences are due simply to the fact that one is physically bound to the contact membranes while the other is free in the cytoplasm. One final difference between the stationary and the relic nuclei is seen in the fact that fertilization takes place between the incoming migratory nucleus and the stationary nucleus even if the relic nuclei are in the same cytoplasm. Perhaps it is premature, in the absence of genetic evidence, to conclude that the migratory nucleus *never* fuses with a relic nucleus, but this conclusion is certainly strongly indicated for other organisms that have been studied genetically (Sonneborn, 1947). It appears probable that the stationary nucleus is

attracted to or attracts the migratory nucleus and that the nuclei are so differentiated that under normal conditions the relic nuclei cannot participate in the union.

There is apparently no major difference between the fertilization nucleus and one of its daughter nuclei. Either may divide once to give rise to a macronucleus and a micronucleus. The difference in their normal behavior is apparently due to their cytoplasmic location rather than to intrinsic factors. The differences in the sizes of the macronuclei and micronuclei produced directly by these two kinds of nuclei are not understood, but may be explained on the basis of a limitation of substrate for nuclear development in the conjugating cells. According to this view, a single macronucleus and a single micronucleus in the cytoplasm have more reserves to draw on and hence develop further than would two macronuclei and two micronuclei in the same cytoplasm. Other interpretations are possible, however.

The disintegration of the macronucleus at a particular time in the conjugation cycle may also be considered a type of nuclear differentiation, but no information is available concerning the factors involved.

The final nuclear differentiation is that which distinguishes the macronuclei from the micronuclei. The evidence presented demonstrates that this differentiation of nuclei is directly related to their positions in the cytoplasm at a critical time. The conditions at the anterior end of the cell are such as to bring about the development of macronuclei; the conditions at the posterior end cause the development of micronuclei. That the nuclei developing as macronuclei are not different in their potentialities from those developing as micronuclei is shown by the fact that presumptive macronuclei may be induced to become micronuclei and presumptive micronuclei may be induced to become macronuclei by altering the positions of the nuclei in the cytoplasm. This conclusion is further supported by evidence that under some circumstances the daughter nuclei produced at the first post-zygotic division can directly differentiate as macronuclei or micronuclei; under normal circumstances each of these nuclei gives rise to one macronucleus and one micronucleus. It would be difficult to explain these results on the basis of the segregation of genetic elements.

4. *Cytoplasmic differentiation*

Evidence for nuclear differentiation of various kinds is available in the account given above. Evidence for progressive cytoplasmic differentiation through conjugation is less readily obtained, but certain observations suggest that this also plays an important role. It is known, for example, that a diploid nucleus dividing immediately anterior to the old macronucleus undergoes meiosis during the initial stages of conjugation, but undergoes mitosis after fertilization in the same position. This could be explained by some kind of nuclear differences characterizing the nuclei at the different times, but it is equally possible that the cytoplasm has been altered. Similar considerations hold in regard to the behavior of nuclei at the anterior end of the cell at different times during conjugation. The nuclei produced after the second pre-zygotic division show no evidence for developing as macronuclei, while the nuclei produced after the second post-zygotic division and placed at the anterior end do develop as macronuclei. Other examples could also be drawn in which nuclear and cytoplasmic differentiation are equally probable as an explanation for the differences in the behavior of nuclei in the same cytoplasmic re-

gions at different times. It appears probable that a progressive cytoplasmic alteration is correlated with a progressive nuclear alteration throughout conjugation and that an understanding of the process must include consideration of a complex interaction of nuclear and cytoplasmic factors.

Although the observations on *Tetrahymena* demonstrate an important influence of the cytoplasm on nuclear behavior, it cannot be concluded that the cytoplasmic conditions are not ultimately under the control of the nuclei. Observations on *Paramecium* (Sonneborn, 1951; Nanney, 1953) indicate that certain cytoplasmic conditions controlling nuclear development are determined by the nuclei and that cellular differentiation may proceed as a series of inter-determinations of the nucleus by the cytoplasm and of the cytoplasm by the nucleus. It is probable that many aspects of nuclear behavior, though immediately under the control of the cytoplasm, are ultimately traced to nuclear activity.

## Summary

1. The details of the nuclear processes occurring at conjugation in certain selfing strains (AA strains) of *Tetrahymena* are presented with an experimental analysis of certain of the factors influencing nuclear behavior and nuclear differentiation.

2. While it is not possible at the present time to describe in detail the mechanisms operating to assure an orderly sequence of events, it is clear that the cytoplasm plays a critical role in directing the activities of the nuclei. This is demonstrated by two facts: that the various events are specifically localized in the cytoplasm and that experimental alterations in the positions of the nuclei result in alterations in nuclear behavior.

3. It is concluded that the entire conjugation cycle proceeds as a complex series of nucleo-cytoplasmic interactions.

## LITERATURE CITED

Chen, T. T., 1946. Conjugation in *Paramecium bursaria*. I. Conjugation of three animals. *J. Morph.*, **78**: 353–395.

Corliss, J. O., 1952. Systematic status of the pure culture ciliate known as "*Tetrahymena geleii*" and "*Glaucoma piriformis*." *Science*, **116**: 118–119.

Elliott, A. M., and D. F. Gruchy, 1952. The occurrence of mating types in *Tetrahymena*. *Biol. Bull.*, **103**: 301.

Elliott, A. M., and D. L. Nanney, 1952. Conjugation in *Tetrahymena*. *Science*, **116**: 33–34.

Furgason, W. H., 1940. The significant cytostomal pattern of the "Glaucoma-Colpidium" group, and a proposed new genus and species, *Tetrahymena geleii*. *Arch. f. Protist.*, **94**: 224–266.

Maupas, E., 1889. Le rajeunissement karyogamique chez les cilies. *Arch. Zool. exp. gen., Ser. 2*, **7**: 149–517.

Nanney, D. L., 1953. Mating type determination in *Paramecium aurelia*, a model of nucleo-cytoplasmic interaction. *Proc. Nat. Acad. Sci.*, **39**: 113–119.

Preer, J. R., 1950. Microscopically visible bodies in the cytoplasm of the "killer" strains of *Paramecium aurelia*. *Genetics*, **35**: 344–362.

Sonneborn, T. M., 1947. Recent advances in the genetics of *Paramecium* and *Euplotes*. *Adv. in Genetics*, **1**: 263–358.

Sonneborn, T. M., 1950a. Methods in the general biology and genetics of *Paramecium aurelia*. *J. Exp. Zool.*, **113**: 87–148.

Sonneborn, T. M., 1950b. The cytoplasm in heredity. *Heredity*, **4**: 11–36.

Sonneborn, T. M., 1951. Some current problems of genetics in the light of investigations on *Chlamydomonas* and *Paramecium*. *Cold Spring Harb. Symp. Quant. Biol.*, **16**: 483–503.

Weisz, P. B., 1950. Multiconjugation in *Blepharisma*. *Biol. Bull.*, **98**: 242–246.

# FRESH-WATER POLYCHAETOUS ANNELID, MANAYUNKIA SPECIOSA LEIDY, FROM LAKE ERIE

MARIAN H. PETTIBONE

*Zoology Department, University of New Hampshire, Durham, New Hampshire*

A collection of some 23 specimens of a very small fresh-water sabellid, collected in Lake Erie by George M. Moore, was presented to me for examination. They proved to be specimens of the interesting fresh-water sabellid polychaete genus, *Manayunkia* Leidy, which is very closely related to *Fabricia* Blainville consisting of marine species. There has been some confusion as to whether one or two species are involved—*M. speciosa* Leidy, 1858, and *M. eriensis* Krecker, 1939.

*Manayunkia speciosa* was originally described from specimens obtained from the Schuylkill River at Fairmount, Philadelphia (Leidy, 1858, 1883)—the first record of a strictly fresh-water polychaete. They were found in tubes of mud attached to stones. Additional specimens were found in Egg Harbor River, New Jersey, attached to a fragment of pine-bark. Further observations were made by Potts (1884) and Foulke (1884).

Meehean (1929) found specimens of *Manayunkia* dredged in the western end of Lake Superior (Duluth Harbor). Under the name of *M. speciosa*, he added to the original description, principally in regard to the collarette; he observed only three pairs of haemal loops, one pair in each of the last three segments, instead of a pair in each segment as indicated by Leidy. Krecker (1939) found specimens of *Manayunkia* in Lake Erie, 30 miles due east of Put-in-Bay, at a depth of 55 feet. He considered that the specimens from Lake Erie were the same species as those from Lake Superior but differed sufficiently to be considered distinct from *M. speciosa* and gave the name of *M. eriensis* for the Great Lakes specimens. Hartman (1951), in a review of the subfamily Fabriciinae, indicated that *M. eriensis* Krecker might be the same as *M. speciosa* Leidy. Zenkevitsch (1925), in a detailed study of *M. baicalensis* (Nusbaum) from Lake Baikal, pointed out the general characters of the group to which *Manayunkia* belongs, as well as some errors in the original description of *M. speciosa*.

Based on a study of the Lake Erie specimens, there seems to be only one species involved and *M. eriensis* should go into synonymy. There follows a summary of the description. The specimens are deposited in the United States National Museum (Cat. No. 24736).

## FAMILY SABELLIDAE
## SUBFAMILY FABRICIINAE
## TRIBE THORACOGENEATA ZENKEVITSCH, 1925
## GENUS MANAYUNKIA LEIDY, 1858
## MANAYUNKIA SPECIOSA LEIDY, 1858

**Figure 1, A–K**

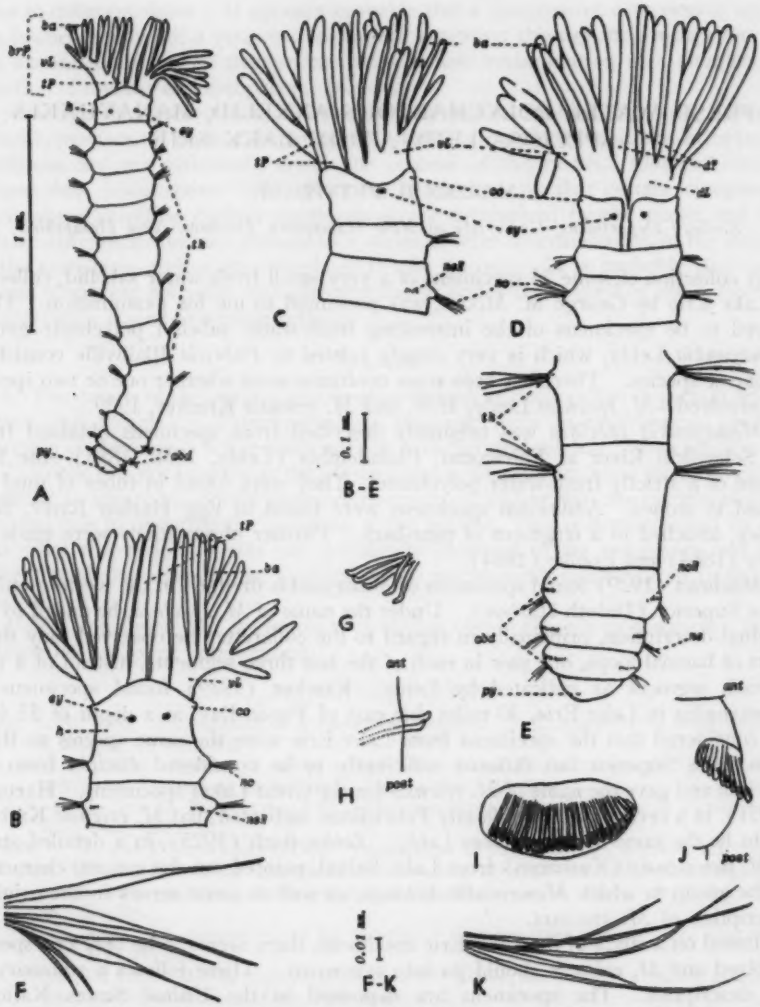



FIGURE 1. *Manayunkia speciosa* from Lake Erie: A, ventral view of entire worm; B, ventral view anterior end; C, lateral view anterior end; D, dorsal view anterior end; E, dorsal view posterior end; F, thoracic notosetae; G, thoracic neuropodial hooks; H, same, showing position in body; I, abdominal notopodial hooks; J, same, showing position in body; K, abdominal neurosetae. abd, abdominal setigerous segments; ant, anterior; ba, ciliated barbules or tentacles; brP, branchial plume or prostomial tentacular crown; co, collarette; dL, dorsolateral inner branch of lateral lobe; dT, mediodorsal tentacles; ey, eyespot; h, "head"—fused prostomium and achaetous first segment; ne, neurosetae or ventral setae; neH, neuropodial hooks; no, notosetae or dorsal setae; noH, notopodial hooks; post, posterior; py, pygidium or anal segment; th, thoracic setigerous segments; tP, tentacular palps; vL, ventrolateral outer branch of lateral lobe.

*Manayunkia speciosa* Leidy, 1858: 90; 1883: 204–212, Pl. 9, Figs. 1–13.—Potts, 1884: 21–22.—Foulke, 1884: 48–49.—Zenkevitsch, 1925: 36–38.—Meehean, 1929: 479–480.—Hartman, 1951: 389.

*Manayunkia eriensis* Krecker, 1939: 153.—Hartman, 1951: 389.

*Size.* Length 2.5 to 4.9 mm., width up to 0.3 mm. In life, Lake Erie specimens 2.8 to 3.3 mm. long. In a preserved specimen 2.5 mm. long, length of branchial plume is 0.4 mm.; head and thoracic region, 1.85 mm.; abdominal region and pygidium, 0.25 mm.; greatest width, 0.3 mm.

*Description.* Body very small, almost cylindrical, slightly attenuated posteriorly, with segments few in number (12 segments—first achaetous, 8 thoracic setigerous, three abdominal setigerous; Fig. 1, A). Prostomial tentacular crown or branchial plume (Fig. 1, A–D) consisting of : (1) a pair of semicircular lateral lobes (lateral lophophores of Leidy), each pair branching near the base—a diagonal ventrolateral outer branch with four pairs of ciliated barbules or tentacles and a diagonal dorsolateral inner branch with 5 pairs of ciliated barbules; barbules on both branches longest ventrally (basal end of branch), gradually become shorter in more dorsal position; barbules reach about the same distal level—as if they had been cut off; (2) a pair of mediodorsal tentacles; (3) a pair of medioventral tentacles—the tentacular palps, which are slightly larger than and subequal in length to the other tentacular appendages; they receive branches of the blood vascular system (the only ones of the tentacular prostomial appendages that have blood vessels); in life, they are conspicuous by the bright green blood pulsating rhythmically, contracting and expanding longitudinally, thus alternatingly contracting and dilating the blood vessels. Thus, there are 20 ciliated tentacles on each side, 40 altogether in the branchial plume. [Leidy, in the original description, indicated that the lateral lophophores were simple, not bilobate; this was questioned by Zenkevitsch (1925, p. 37), since in *M. aestuarina* and *M. baicalensis* each lophophore is bilobed; close examination shows that this is also the case in *M. speciosa*.] There may be 6–10 brownish pigment spots on each side near the bases of the barbules (not visible when preserved; according to Meehean, younger ones have few or none; Leidy, Pl. 9, Fig. 1).

Prostomium fused with first achaetous segment (conveniently referred to as the "head"), with a pair of eyespots near the bases of the tentacular palps, with a distinct collarette—on the ventral side, projected anteriorly into a rounded lobe; open and separated middorsally; collarette may be somewhat flared (Fig. 1, B–D; Leidy made no mention of a collarette although the ventral extension of it was shown on his Fig. 1; it was noted by Meehean). Anterior thoracic region consists of 8 setigerous segments—first with dorsal setae only (notosetae), remainder with additional long-handled ventral uncini or podal hooks (neuropodial hooks). Thoracic notosetae 5–8 in number, limbate, with capillary tips (Fig. 1, F; Leidy, Pl. 9, Figs. 3–4). Thoracic neuropodial hooks 3–5 in number, with long curved handle, ending in small recurved hook directed anteriorly (Fig. 1, G–H; Leidy, Pl. 9, Fig. 5). Posterior abdominal region consists of last three setigerous segments (Fig. 1, E), with dorsal long-handled uncini or podal hooks (notopodial hooks) and ventral setae (neurosetae). Abdominal notopodial hooks consisting of numerous (14–30) small hooks arranged in a close transverse row; hooks with broad handle ending in expanded pectinate tip—numerous very fine teeth in several rows on one side, directed anteriorly (Fig. 1, I–J; Leidy, Pl. 9, Figs. 6–7). Abdominal neuro-

setae 3–5 in number, slender, with capillary tips (Fig. 1, K). Anal segment or pygidium short, rounded, without eyespots (Fig. 1, E).

Food brought to funnel-like, capacious mouth by ciliary currents of the branchial barbules. Digestive tract a median tube, alternately dilated within the segments, ciliated for the whole length (Leidy, Pl. 9, Fig. 1). Anus opens ventrally between last segment and pygidium. Excrement passed forward in the tube, aided by the longitudinal ciliated groove—midventral in abdominal region, passing diagonally on one side, middorsal in thoracic region. Blood vascular system, containing green blood, consists of a sinus around the digestive tract (corresponds to the dorsal blood vessel) in which the blood passes anteriorly by contractions of the gut wall, two branchial "hearts" at the bases of the branchial lobes, two vessels in the tentacular palps, a ring of vessels near the mouth, a vessel ventral to the digestive tract, paired loops in each segment from the ventral blood vessel to the intestinal sinus, the last three pairs especially conspicuous. A pair of looped nephridial tubes in segments 1–2 (these paired large elliptical organs were suspected by Leidy to be the testes, although he stated that he did not examine their structure).

Sexes separate (not hermaphroditic as indicated by Leidy; see Zenkevitsch, 1925). Females with ovaries and eggs in different stages of development in segment 5 (setigerous segment 4); eggs escape through opening between segments 5–6 (Meehean, 1929). Eggs laid within the tube where they pass their early developmental stages; young retained until they have at least 8–9 setigerous segments, with eyespots present, with 4–5 pairs of barbules on the branchial plume (Leidy, Pl. 9, Figs. 8–13). On the mature females, there may be a thickened semicircular band on the ventral side of the anterior part of segment 7 (setigerous segment 6; this expansion of the forepart of segment 7 suggested to Leidy that it might be the production of a head prior to the transverse fission of the worm, although he did not observe the process; Pl. 9, Fig. 1; Zenkevitsch showed that it was present only in mature females and formed part of a brood chamber between segments 7 and 9). Males with testes and sperm in segments 7–9 (setigerous segments 6–8; not in segments 1–2 as indicated by Leidy—these structures proved to be the paired nephridia as indicated above). (In *M. baikalensis*, a mediodorsal sperm duct passes from segments 6–9, conducting the sperm to the head region; opens by genital pore near the base of the head behind the nephridiopore; the anterior part of the sperm duct with distinct enlargement filled with sperm, forming a seminal vesicle; details not worked out for *M. speciosa*.)

Color (preserved): without color or may be darkly pigmented in part of head segment; (living): translucent olive-green, slightly brownish around the branchial lobes. Tube of mud—fine particles agglutinated by mucoid secretion, feebly annulated, cylindrical, straight or bent, sometimes branched (may be 2–5 branches), attached to fixed objects, the greater part free, pendant; tube may reach a length of several times that of the worm. (The specimens from Lake Erie were out of their tubes but some small empty tubes were found in the same haul.)

*Locality.* Lake Erie, 22 miles east of Put-in-Bay, Ohio, depth of 55 feet, July 13, 1951, collected by George M. Moore. Dredged by fine screened trawl on bottom of gray clayish mud with numerous dead shells. Some 23 specimens found in washings from the dredge, along with hydras, planarians, oligochaetes, and snails.

*Distribution.* North American fresh waters: rivers of southeastern Pennsyl-

vania (Schuylkill River, Philadelphia) and southeastern New Jersey (Egg Harbor River); Great Lakes—Lake Erie (near Put-in-Bay, Ohio, 55 feet) and Lake Superior (Duluth Harbor, Minnesota, dredged). Probably much more widely distributed but, due to its small size, escapes notice.

## LITERATURE CITED

FOULKE, S. G., 1884. Some notes on *Manayunkia speciosa*. *Proc. Acad. Nat. Sci. Phil.*, **36**: 48–49.

HARTMAN, O., 1951. Fabricinae (feather-duster polychaetous annelids) in the Pacific. *Pacific Sci.*, **5**: 379–391.

KRECKER, F. H., 1939. Polychaete annelid worms in the Great Lakes. *Science*, **89**: 153.

LEIDY, J., 1858. *Proc. Acad. Nat. Sci. Phil.*, **10**: 90.

LEIDY, J., 1883. *Manayunkia speciosa*. *Proc. Acad. Nat. Sci. Phil.*, **35**: 204–212.

MEEHEAN, O. L., 1929. *Manayunkia speciosa* (Leidy) in the Duluth Harbor. *Science*, **70**: 479–480.

POTTS, E., 1884. Note on *Manayunkia speciosa*. *Proc. Acad. Nat. Sci. Phil.*, **36**: 21–22.

ZENKEVITSCH, L. A., 1925. Biologie, Anatomie und Systematik der Süsswasserpolychaeten des Baikalsees. *Zool. Jahrb., Abt. f. System.*, **50**: 1–60.

# SOME ASPECTS OF PHYSIOLOGICAL AGING IN THE ADULT WORKER HONEY BEE [1, 2]

MORRIS ROCKSTEIN

*Laboratories of Zoophysiology, State College of Washington, Pullman, Washington*

In reviews on the general physiology of aging, Lansing (1947, 1951) has emphasized the need for study at a cellular level of the problem of senescence, a phenomenon demonstrating a uniform pattern in a wide variety of organisms. This concept encompasses examination of anatomical and morphological, degenerative alterations, as well as biochemical changes such as hormonal and enzymic perturbations accompanying the involutionary process of growing old. Such studies must necessarily involve a long-term investigation of the various facets of the phenomenon of aging in animals or individuals whose complete medico-ecological history is a matter of record. To this end the worker honey bee (*Apis mellifera*) represents an animal which can be obtained in large numbers and which can be maintained during its life span under controlled physical environmental conditions with a minimum of care, while occupying relatively little space.

In an earlier study, Rockstein (1950) reported that the number of cells at two representative levels of the brain of the adult worker bee decreased steadily from the day of emergence to old age, whereas the activity in total brain homogenates of the enzyme cholinesterase rose during the first week to ten days following emergence and remained at this elevated level throughout the remainder of the life of the bee. This indicated the absence of a significant role by this enzyme system in the physiology of aging, from the standpoint of senescence.

Important in many aspects of intermediary metabolism, specifically in vital processes like nucleic acid and carbohydrate metabolism (see Moog, 1946 and Roche, 1950), the phosphomonoesterases suggested another enzyme system of sufficiently elevated importance which might prove related to the process of senescence. In pursuing this problem further, the writer has therefore studied changes in activity of acid and alkaline sodium $\beta$-glycerophosphatases in total body homogenates, as a function of age in the adult worker honey bee, *Apis mellifera* L., the results of which studies are presented herewith.

## Methods

Frames of sealed worker brood were removed from a colony in the college apiary to an incubator maintained at 32.5° C. Adult bees in large numbers (2000 or more) were removed within 24 hours after emergence and were maintained

[1] This investigation was supported in part by funds provided for biological and medical research by the State of Washington Initiative Measure No. 171.

[2] The author is indebted to Mr. Leonard Levine and Mr. M. David Inashima for technical assistance and to Mr. Don A. Belden, Jr. for his assistance in preparing some of the data for publication. He acknowledges also the generous cooperation of Dr. H. S. Telford and Mr. H. Menke in making available live material for this study.

thereafter in a large screen cage, under conditions of continuous artificial lightining and at a constant temperature of 26.5° C. and relative humidity of 50%, with adequate pollen, honey and water supplies for *ad libitum* feeding. At definite intervals of time, bees of known age were removed and homogenized in lots of twenty by the procedure described by Rockstein and Herron (1951), and the acid and alkaline phosphatase activity determined, respectively, as described by Rockstein and Levine (1951) and Rockstein and Inashima (1953). Determinations on very old bees were deferred until the point where the cage population began to show a rapid decline and where just enough live material was available.

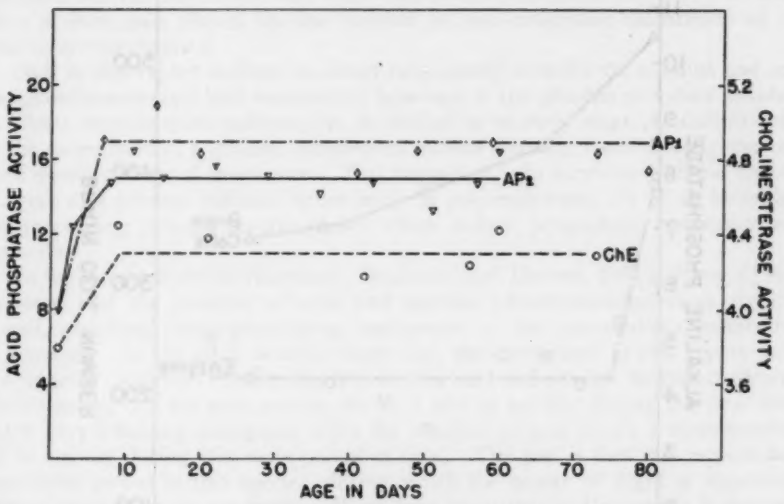



FIGURE 1. Acid phosphatase in whole body and cholinesterase in whole brain homogenates of the adult worker honey bee.

## RESULTS

Figure 1 shows two curves labelled "$AP_1$" and "$AP_2$", denoting acid phosphatase studies of whole body homogenates for two populations of bees, as compared to data from the earlier report by the author (1950), averaged and plotted for cholinesterase activity in whole brain homogenates. Phosphatase activity is expressed as micrograms of phosphate as phosphorus, released in 1½ hours at 35° C. at pH 5.4, in a 0.2 ml. sample of deproteinized incubation mixture (see Rockstein and Levine, 1951, for details); cholinesterase activity is expressed as micromoles of acetylcholine bromide hydrolyzed per whole brain per hour at 30° C. During the first eight to ten days of adult life the total body acid enzyme shows a rise in activity representing an increase of about 90%; during the corresponding period the total brain cholinesterase shows a parallel rise in activity, but of only 14%. For both enzymes this elevated activity appears to remain undiminished from about ten days to very old age.

Figure 2 shows similar curves for the alkaline enzyme (only one series) and collated data for brain cell number, from the author's earlier report (1950), averaged and plotted for comparison. Alkaline enzyme units are the same as for the acid enzyme, representing activity under identical conditions except that the pH of incubation was maintained at 8.1. Here is seen a steady decrease in brain cell number to the extent of about a 35% decline in old bees from the original cell count in day-old bees; total body alkaline enzyme, however, shows a steady decline

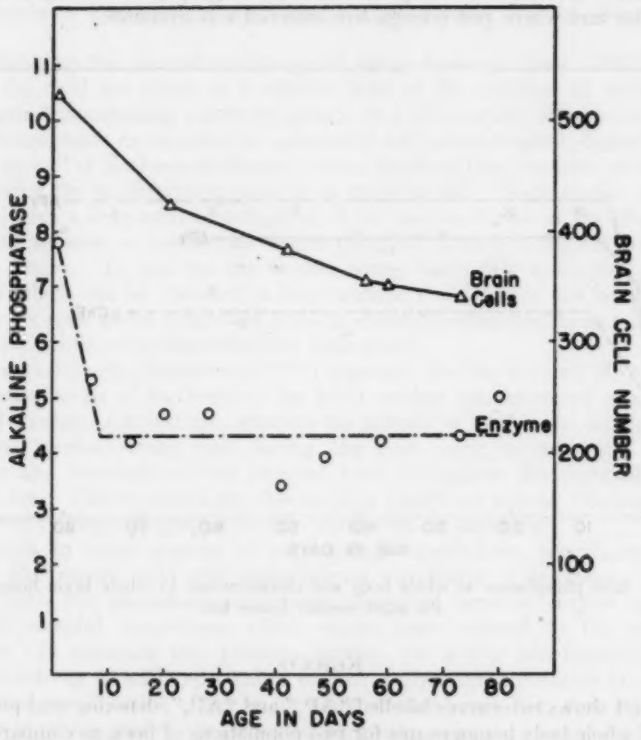



FIGURE 2. Alkaline phosphatase in whole body homogenates and brain cell number in the adult worker honey bee.

during the first week to ten days to a level 44% below that of day-old bees, following which no significant change occurs until old age.

Each point plotted for phosphatase activity in both figures represents the median value for at least five replicate determinations from each homogenate preparation.

## DISCUSSION

If the total aging process is accepted to be an outward manifestation of inner biochemical alterations at the cellular level, the immediately controlling process or

processes may be more precisely defined in terms of modifications in the activity of identifiable enzyme systems catalyzing important metabolic processes. One agrees that correlation of data, whether it be statistically or graphically demonstrable, does not guarantee causality for phenomena under consideration, biological or otherwise. However, as in the case of cholinesterase, an enzyme important in the metabolism of acetylcholine (itself important in the mediation of the nerve impulse in certain parts of the nervous system of most animals), deductions can be made with some degree of certainty concerning their rationality.

From the earlier study by Rockstein (1950), no such positive relationship was indicated between senescence and brain cholinesterase in the honey bee, but rather some positive part played by that enzyme in post-embryonic maturation of the neuromotor mechanism.

Data in this report indicate no direct relationship between the alkaline and acid phosphomonoesterases and senescence; however, if the alkaline is indeed involved its effects must be quite indirect; *i.e.*, its decline at an early stage of adult life may be the forerunner of a gradual biochemical decline eliciting more directly the outward manifestations of senescence. This suggestion is in harmony with the concept of aging as a process initiated rather early in post-embryonic life of an individual and proceeding through several phases which include progressive maturation and senescence.

As has been mentioned elsewhere (Rockstein and Herron, 1951), Moog (1946) proposed that the presence of acid and alkaline phosphomonoesterases signifies a dual, matching dephosphorylating mechanism in the intermediary metabolism of glycogen. In the adult worker honey bee, data presented in this report point to a singular reciprocal relationship between the acid and alkaline sodium $\beta$-glycerophosphatases; *i.e.*, the acid enzyme shows a rise in activity during the first week to ten days following emergence while the alkaline enzyme shows a corresponding fall in activity during this same period of time. The fact is that this represents a post-larval period in this species, during which the power of flight is apparently being developed to its maximum. Wing beat frequency in *Drosophila* is reported by Sacktor (1950) as having been found by Chadwick and Williams (unpublished data) to be low immediately following emergence and to increase during the first few days of adult age to a level maintained throughout adult life; Sacktor himself found a marked increase in the activity of cytochrome oxidase, in total body homogenates of DDT-resistant and normal strains of the common house fly, the second day after adult emergence. The fact that brain cholinesterase in the adult honey bee also shows a strikingly parallel increase during the first week to ten days of adult life suggests that the interrelationship among the three enzyme systems, mentioned for the honey bee in this report, may be part of a well-integrated pattern of biochemical alterations concerned with completion of development of adult characteristics, particularly the neuromotor mechanism of strong-flying species. In this connection, evidence has been presented in an earlier discussion by Rockstein (1950), as well as in reports by Nachmansohn (1939), Sawyer (1943a, 1943b, 1944), Welsh and Hyde (1944) and Lindeman (1945), concerning the correlation between cholinesterase activity and the attainment of the ability to perform rapid movements in whole embryos, in developing immature young, and in different species of animals with varying degrees of motor ability.

Watanabe and Williams (1951) reported that the cytochrome oxidase activity in isolated sarcosomes (giant mitochondria) of the flight muscles of *Phormia regina* showed a pronounced drop (about 50%) during the first three to four days following adult emergence, at which reduced level of activity the enzyme in question remained constant thereafter. The variance between their data and those of Sacktor (1950) for total homogenates of the house fly indicates a need for further study of this enzyme in the house fly at the tissue level. Watanabe and Williams also reported a precipitous decline in catalase activity of sarcosomes during the similar three-four day period following emergence to about 20% of the original activity of a newly emerged adult fly, which decline was followed by a gradual dropping off to a low level of about 10% of the original activity at the end of the third week of adult life. The pattern of the latter findings suggests a possible basis for re-examination of the biochemical picture in the honey bee with regard to catalase, as well as the phosphatases, at the organ or tissue level, for possible further clarification of the maturation process during the post-emergence period in this species, and in other insects with a strong flight pattern.

## Summary

1. Acid sodium $\beta$-glycerophosphatase in whole body homogenates of adult worker honey bees shows a rise in activity by about 90% from the first to the tenth day following emergence and remains unchanged thereafter at this elevated level until old age.

2. Alkaline phosphatase shows a steady decline in activity to about 44% below that of day-old bees, by about the tenth day following emergence, remaining unchanged at the lowered level until old age.

3. Although no direct relationship between these enzymes and the process of senescence is apparent, the correspondence between these data and earlier findings is discussed in terms of the post-emergence development of the neuromotor mechanisms in vigorous-flying holometabolous insects.

## LITERATURE CITED

Lansing, A. I., 1947. The general physiology of aging—a review. *J. Gerontol.*, **2**: 327–338.

Lansing, A. I., 1951. Some physiological aspects of ageing. *Physiol. Revs.*, **31**: 274–284.

Lindeman, V. F., 1945. A comparative study of the cholinesterase activity of the vertebrate nervous system with special reference to its relationship to motor ability. *Amer. J. Physiol.*, **143**: 687–691.

Moog, F., 1946. The physiological significance of the phosphomonoesterases. *Biol. Revs.*, **21**: 41–59.

Nachmansohn, D., 1939. Cholinestérase dans le système nerveux central. *Bull. Soc. Chim. Biol.*, **21**: 761–796.

Roche, J., 1950. Phosphatases. *In:* The enzymes, Ed. by J. B. Sumner and K. Myrbäck. Academic Press. Chapter 11.

Rockstein, M., 1950. The relation of cholinesterase to change in cell number with age in the brain of the adult worker honeybee. *J. Cell Comp. Physiol.*, **35**: 11–24.

Rockstein, M., and P. W. Herron, 1951. Phosphatase in the adult worker honey bee. *J. Cell. Comp. Physiol.*, **38**: 451–467.

Rockstein, M., and M. D. Inashima, 1953. Enzymes in insects. Alkaline phosphatase. *Bull. Brooklyn Entomol. Soc.*, **48**: 20–23.

Rockstein, M., and L. Levine, 1951. Enzymes in insects. Acid phosphatase. *Annals Entomol. Soc. Amer.*, **44**: 469–472.

SACKTOR, B., 1950. A comparison of the cytochrome oxidase activity of two strains of house flies. *J. Econ. Entomol.*, **43**: 832–838.

SAWYER, C. H., 1943a. Cholinesterase and the behavior problem in Amblystoma. I. The relationship between the development of the enzyme and early motility. II. The effects of inhibiting cholinesterase. *J. Exp. Zool.*, **92**: 1–29.

SAWYER, C. H., 1943b. Cholinesterase and the behavior problem in Amblystoma. III. The distribution of cholinesterase in nerve and muscle throughout development. IV. Cholinesterase in nerveless muscle. *J. Exp. Zool.*, **94**: 1–31.

SAWYER, C. H., 1944. Nature of the early somatic movements in *Fundulus heteroclitus*. *J. Cell. Comp. Physiol.*, **24**: 71–84.

WATANABE, M. I., AND C. M. WILLIAMS, 1951. Mitochondria in the flight muscles of insects. I. Chemical composition and enzymatic content. *J. Gen. Physiol.*, **34**: 675–689.

WELSH, J. H., AND J. E. HYDE, 1944. The distribution of acetylcholine in brains of rats of different ages. *J. Neurophysiol.*, **7**: 41–49.

# EMBRYONIC DEVELOPMENT OF THE LORDOTIC AND NORMAL GUPPY, LEBISTES RETICULATUS (PETERS) [1]

HAROLD L. ROSENTHAL

*Dept. of Biochemistry, Tulane University of Louisiana, New Orleans 12, Louisiana*

Previous studies have shown that the mutation resulting in lordosis of the vertebral column in *Aplocheilus latipes* (Aida, 1930) and in *Lebistes reticulatus* (Kirpichnikov, 1935; Harrison, 1941; Goodrich *et al.*, 1943; Rosenthal and Rosenthal, 1950) behaves as a recessive, autosomal single factor Mendelian character. The lordotic condition becomes progressively more pronounced as the fish ages and may cause swimming activity to be somewhat laborious and erratic. Growth and development of either sex and reproduction in the female do not appear to be affected. However, the fertilizing capacity of sperm from mature mutant males is reduced (Rosenthal, 1951).

Since the spinal curvature is markedly apparent in the new-born young of *Lebistes*, it was of interest to compare the growth and development of the lordotic and normal embryo as an introduction to the study of the biochemistry of the lordotic mutation.

## Materials and Methods

Normal fish, obtained from a local tropical fish hatchery,[2] were maintained in the laboratory for variable lengths of time before use. The lordotic fish were raised and maintained under conditions similar to those previously reported (Rosenthal and Rosenthal, 1950; Rosenthal, 1952). As female fish approached parturition, they were isolated to individual jars containing masses of *Ceraptopterus* until the brood was born. The new-born young were removed within 3 to 20 hours, blotted dry on filter paper and the entire brood weighed to the nearest 0.1 mg. The young were then dried to constant weight at 105° C. to determine their water content. The dry residue was next extracted with ten 3-ml. portions of ethyl ether and the fat-free residue was dried an additional 24 hours. The fat content of the material was calculated by difference. A series of embryos [3] was obtained by sacrificing female fish at weekly intervals following the birth of a brood. The fish were killed by dropping them into boiling water since heat coagulation was found to be the best method for removing the ova or embryos from the ovary without breaking them. The ovarian contents were treated in the same manner

[1] Supported in part by a grant in aid from the Society of Sigma Xi-Resa Research Fund.

[2] Normal fish and live food such as Tubifex worms were generously supplied by Messrs. William Schaumberg and John Detrich, of Crescent Fish Farms, New Orleans, to whom the author is indebted.

[3] Since the embryo cannot be readily separated from the yolk of the egg, the entire embryo-yolk complex was removed and treated as a unit and this combination of embryo and yolk will be considered interchangeably, for the purposes of this report, as either the ovum, embryo-yolk complex or embryo depending on the data to be discussed.

as new-born young. Embryos or young that were not normally developed were discarded.

For chemical analysis, quantities of dried, fat-free material weighing 15 to 40 mgs. from individual broods of young or groups of ova and embryos were digested by boiling with one ml. concentrated sulfuric acid containing 4 drops of 5% copper sulfate. The digests were completely cleared by the addition of a few drops of 30% hydrogen peroxide. After cooling, the digests were diluted to 10 ml. and suitable aliquots were taken for the determination of calcium by the method of Kramer and Tisdall (1921). Phosphorus was determined on aliquots of the digest by the method of Fiske and Subbarow (1925) and nitrogen by direct nesslerization. Nitrogen values were converted to protein by use of the factor 6.25. Duplicate determinations of calcium agreed within 6% and those for phosphorus and nitrogen within 1%.

## Results

A comparison of the data, shown in Table I, on the growth rate, fat and water content of the normal and lordotic *Lebistes* indicates no essential differences with the exception that the lordotic embryo is consistently the smaller. In view of the similarity between the two strains, the data will be discussed together.

Although the variation between groups of embryos is quite large as shown by the standard deviations, it can be seen that the wet weight of maturing ova remains essentially constant for the first seven days but increases rapidly from the time of fertilization until the embryo is born. The increase in weight is due to an in-

TABLE I

*Embryonic growth of Lebistes reticulatus*

| Age, days | No. of embryos | No. of deter. | Average weight (mgs.) | | | $H_2O$ % wet wt. | Fat % dry wt. |
|---|---|---|---|---|---|---|---|
| | | | Wet | Dry | Fat free | | |
| **Normal** | | | | | | | |
| 0 | 185 | 5 | 2.96±0.73* | 1.46±0.36* | 1.14±0.20* | 50.6 | 21.6 |
| 7 | 154 | 6 | 2.75±0.40 | 1.31±0.28 | 1.01±0.19 | 53.1 | 22.5 |
| 14 | 187 | 6 | 3.86±0.57 | 1.33±0.33 | 1.01±0.31 | 70.9 | 24.3 |
| 21 | 178 | 7 | 4.95±0.44 | 1.24±0.25 | 0.95±0.11 | 75.0 | 23.4 |
| Term | 279 | 23 | 5.64±0.69 | 1.22±0.20 | 0.96±0.15 | 78.4 | 19.5 |
| **Lordotic** | | | | | | | |
| 0 | 47 | 4 | 2.06±0.61 | 1.00±0.28 | 0.73±0.23 | 51.6 | 26.7 |
| 7 | 85 | 3 | 2.56±0.12 | 1.23±0.05 | 0.94±0.03 | 52.2 | 23.5 |
| 14 | 92 | 8 | 3.14±0.93 | 1.29±0.21 | 1.02±0.19 | 63.1 | 21.8 |
| 21 | 50 | 3 | 4.06±0.38 | 1.07±0.18 | 0.83±0.15 | 73.8 | 23.2 |
| Term | 108 | 25 | 4.53±0.56 | 0.98±0.20 | 0.84±0.13 | 79.2 | 14.1 |

* $\sigma = \sqrt{\frac{\Sigma d^2}{N-1}}$.

creasing water content which rises from an initial value of 50% to a final value of 80%. In contrast, the dry and the fat-free dry weight remain essentially constant throughout the development of the embryo. The fat content of the embryo, however, decreases slightly after the young are born. This probably reflects a utilization of stored yolk material during the period immediately following birth, since newly born poeciliid fish have rarely been observed to accept food during the first few hours after birth.

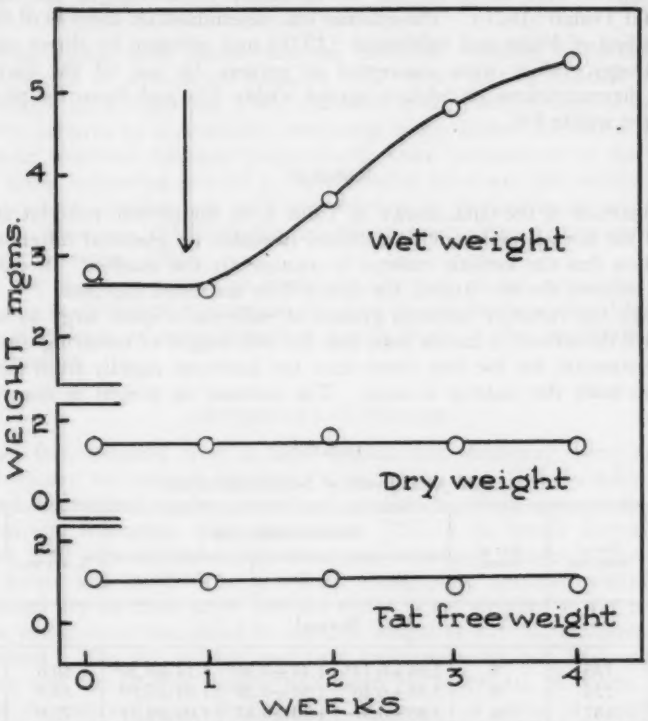



FIGURE 1. Average growth curves for lordotic and normal embryos of *Lebistes* during the brood interval. The arrow indicates the approximate time of fertilization of the ovum. See Table I.

In view of the similarity of the lordotic and normal embryo, the wet, dry and fat-free dry weights have been averaged and plotted against time of development as shown in Figure 1, to demonstrate graphically the growth rate of the *Lebistes* embryo.

calcium and phosphorus content of the dry fat-free tissue during growth and development of the *Lebistes* embryo. It is apparent that the total protein remains

The data presented in Table II show the changes that occur in the nitrogen, essentially constant for both the lordotic and the normal strains although the values

obtained for the lordotic strain are somewhat smaller due to the smaller size of the embryo. The per cent of protein is also the same for both strains and remains essentially constant during gestation. The average total protein of the embryo-yolk complex accounts for 71.1 per cent of the fat-free dry weight (range = 67.1 – 76.1 per cent). The remainder consists of minerals and non-proteinaceous organic matter.

The calcium and phosphorus concentrations of the embryos present a somewhat different picture. During the first three weeks of development, the calcium concentration remains essentially constant but increases rapidly during the last week of gestation. The phosphorus concentration, on the other hand, increases

TABLE II

*Embryonic growth of Lebistes reticulatus*

| Age, days | No. of deter. | Protein | | Calcium | Phosphorus | Ca/P |
|---|---|---|---|---|---|---|
| | | Total mgs. | % Fat-free dry weight | | | |
| | | | *Normal* | | | |
| 0 | 4 | 0.77 | 67.7±3.7* | 0.62±0.21* | 1.18±0.07* | 0.43 |
| 7 | 5 | 0.71 | 70.9±5.0 | 0.81±0.18 | 1.50±0.08 | 0.54 |
| 14 | 6 | 0.70 | 69.7±3.2 | 0.76±0.06 | 1.52±0.07 | 0.50 |
| 21 | 6 | 0.70 | 73.3±2.1 | 1.08±0.29 | 1.60±0.60 | 0.67 |
| Term | 12 | 0.70 | 73.3±3.9 | 2.24±0.40 | 1.90±0.12 | 1.17 |
| | | | *Lordotic* | | | |
| 0 | 2 | 0.50 | 68.6±4.3 | 0.95±0.05 | 1.35±0.05 | 0.70 |
| 7 | 3 | 0.65 | 68.8±0.8 | 0.73±0.04 | 1.46±0.05 | 0.50 |
| 14 | 4 | 0.74 | 72.8±1.8 | 0.97±0.12 | 1.49±0.08 | 0.65 |
| 21 | 3 | 0.63 | 75.8±4.3 | 0.93±0.12 | 1.66±0.09 | 0.58 |
| Term | 8 | 0.64 | 76.1±7.9 | 2.58±0.51 | 1.92±0.24 | 1.37 |

* $\sigma = \sqrt{\frac{\Sigma d^2}{N-1}}$.

slightly throughout the brood interval. It can be seen that the Ca/P increases only slightly during the early phases of gestation but increases rapidly during the last week preceding birth. The increasing calcium content is presumably associated with the incorporation of mineral elements in the osseous tissues of the body.

Since no essential differences between the lordotic and normal strains are evident, the data in Table II have been averaged for graphic presentation (Fig. 2).

## DISCUSSION

The interval between the birth of successive broods of young for most poeciliid fish in general and *Lebistes* in particular approximates a period of 28 to 30 days. A brood interval may be divided into two separate phases. The first phase consists of 5 to 7 days in which the ova mature to a fertilizable stage (Hopper, 1943;

Rosenthal, 1952; Turner, 1937). The remaining 23 to 25 days, the second phase, may be considered as the period of gestation. The cyclical brood production makes it possible to obtain a graded series of embryos in similar stages of development.

It is apparent from these data that no essential differences exist in the rate of growth of the lordotic and normal *Lebistes* embryo as determined by the methods used. However, Harrison (1941) analyzed the total body calcium of adult

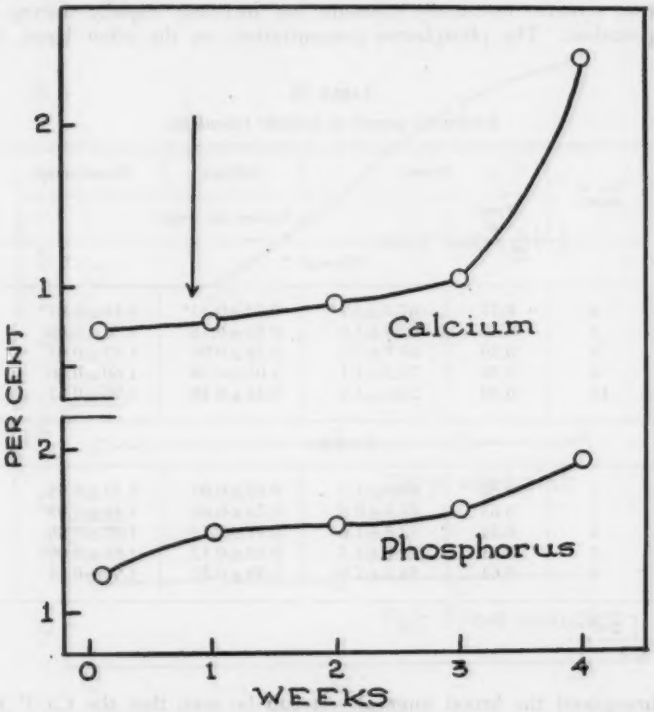



FIGURE 2. Average incorporation of calcium and phosphorus for lordotic and normal *Lebistes* during the brood interval. The arrow indicates the approximate time of fertilization of the ovum. See Table II.

mutant and normal *Lebistes* and found that the mutant strain had a higher calcium content. Additional data (in manuscript) obtained in this laboratory indicate that the vertebral column of the lordotic strain contains about 20% more calcium than the normal.

Scrimshaw (1944; 1945) has studied extensively the embryonic growth pattern of various members of the family Poeciliidae and has concluded, on the basis of the constant dry weight of the embryo yolk complex, that an exchange of nutritive material must occur between the embryo and the tissues of the parent to replace

materials lost through metabolic and excretory processes. The increasing calcium and phosphorus concentrations of the growing embryo, as shown in the present report, indicate that certain materials are obtained from the parent. It is conceivable that organic compounds can also diffuse across the ovarian membranes and, likewise, metabolic products formed during embryogenesis may diffuse through these membranes to be excreted by the parent.

The author wishes to thank Dr. William B. Wendel for his encouragement during the study and his aid in the preparation of this report.

## Summary

1. No differences could be established in the embryonic development of the lordotic and normal *Lebistes*.

2. The water content of developing embryos increases during gestation, but the dry and fat-free dry weights remain constant as does the protein concentration.

3. The calcium concentration, associated with calcification of osseous tissue, increases markedly during the last week of gestation while the phosphorus concentration increases only slightly during the entire brood interval.

## LITERATURE CITED

Aida, T., 1930. Further genetical studies of *Aplocheilus latipes*. *Genetics*, **15**: 1–16.

Fiske, C. H., and Y. Subbarow, 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.*, **66**: 375–400.

Goodrich, H. B., R. W. Harrison, N. D. Josephson and J. P. Trinkaus, 1943. Three genes in *Lebistes reticulatus*. *Genetics*, **28**: 75.

Harrison, R. W., 1941. A contribution to the genetics of *Lebistes reticulatus:* The nature and inheritance of "Hunchback." Thesis. Wesleyan University, Middletown, Connecticut.

Hopper, A. F., Jr., 1943. The early embryology of *Platypoecilus maculatus*. Copeia, No. 3, 180–183.

Kirpichnikov, V., 1935. Autosomal genes in *Lebistes reticulatus* and the problem of the arising of the genetic sex determination. *J. Biol. Moscow*, **4**: 343–354.

Kramer, B., and F. F. Tisdall, 1921. A simple technique for the determination of calcium and magnesium in small amounts of serum. *J. Biol. Chem.*, **47**: 475–481.

Rosenthal, H. L., and R. S. Rosenthal, 1950. Lordosis, a mutation in the guppy, *Lebistes reticulatus*. *J. Hered.*, **41**: 217–218.

Rosenthal, H. L., 1951. Studies on semi-lethalism in the male lordotic guppy, *Lebistes reticulatus*. *J. Hered.*, **42**: 257–259.

Rosenthal, H. L., 1952. Observations on reproduction of the poeciliid *Lebistes reticulatus* (Peters). *Biol. Bull.*, **102**: 30–38.

Scrimshaw, N. S., 1944. Embryonic growth in the viviparous poeciliid fish, *Heterandria formosa*. *Biol. Bull.*, **87**: 37–51.

Scrimshaw, N. S., 1945. Embryonic development in poeciliid fishes. *Biol. Bull.*, **88**: 233–246.

Turner, C. L., 1937. Reproductive cycles and superfetation in poeciliid fishes. *Biol. Bull.*, **72**: 145–164.

# DEPHOSPHORYLATION OF ADENOSINE TRIPHOSPHATE BY TISSUES OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA (L.)

BERTRAM SACKTOR, GERARD M. THOMAS, JOHN C. MOSER AND DONALD I. BLOCH

*Chemical Corps Medical Laboratories, Army Chemical Center, Maryland*

The recent demonstration (Albaum and Kletzkin, 1948; Calaby, 1951) of adenosine triphosphate (ATP) in insects has suggested that this compound may have as important a function in the cellular metabolism of insects as it is known to have in mammals. Although, with the exception of muscle, the significance of dephosphorylation of ATP in mammalian metabolism is still obscure, the resolution of the mechanism of ATP breakdown has been furthered by the partial characterization of this process in other tissues. Analogously, the first reports of dephosphorylation of ATP in insects were with muscle. No doubt contributory insight into this process can be obtained in insects also by a study of the action of other tissues towards ATP.

Compared with the large amount of information available for mammalian tissues, little is known of adenine nucleotide breakdown in insects. Gilmour (1948) noted a Mg-activated soluble enzyme, derived from locust myosin extracts, which split both labile phosphates from the ATP molecule. A similar observation was made by Barron and Tahmisian (1948) with American cockroach muscle homogenates. The effect of temperature on an apyrase in cockroach muscle was studied by Chin (1951). Recently, Gilmour and Calaby (1952) have reported further investigations on the soluble apyrase found in the muscle of locusts. In conjunction with the confirmation that this enzyme was Mg-activated and removed both labile phosphates from ATP, they demonstrated that although traces of adenylate kinase (myokinase) activity were present in their preparation, this mechanism was not responsible for the removal of the second phosphate. Further, the same apyrase could utilize inosine triphosphate (ITP), adenosine diphosphate (ADP) and inosine diphosphate (IDP) as substrates, but was without effect on adenosine monophosphate (AMP) and several other organic phosphates and pyrophosphates. In addition to the apyrase, it was noted that the locust also possesses a Ca-activated ATPase which is associated with the myosin fraction of the muscle.

In contrast to the situation in orthopteran muscle, Sacktor (1953) found other mechanisms of ATP breakdown in the indirect flight muscles of the house fly. Here it was shown that mitochondria isolated from these flight muscles possesssed a specific ATPase, in that they cleaved only the terminal phosphate from ATP and that ATP was the only phosphorylated compound which released inorganic phosphate. Although liberation of orthophosphate also occurred when ADP was the substrate, it was proved that this activity was due to the presence of a Mg-activated adenylate kinase, which converted ADP to ATP and AMP, and thus provided

substrate for the specific ATPase. The mitochondrial ATPase, furthermore, was activated by Mg and Mn but not by Ca ions. On the other hand, an ATPase associated with the muscle *fibrils* was Ca-activated. A soluble fraction of whole thoracic homogenates, like the mitochondria, possessed a Mg-activated ATPase; but this could be distinguished from the mitochondrial ATPase by concurrent inorganic pyrophosphatase activity as well as by differences in the effects of several inhibitors.

The contrast in the mechanism of ATP breakdown between the specialized fibrillar muscle of the house fly and locust muscle leads to the question as to the situation in the other tissues of an insect. Moreover, such investigations should afford a better understanding of the relatively uncomprehended metabolic function of the various insect tissues. In this respect, an approach to a related problem in the comparative physiology of insect tissues was previously reported (Sacktor and Bodenstein, 1952). It was found that different tissues of the American cockroach had different cytochrome *c* oxidase activities which were related to their probable metabolic performance. Furthermore, these results corresponded strikingly with the tracheation of these tissues, as reported by Day (1951). Also, it was discovered that the cytochrome *c* oxidase activity of several tissues was significantly influenced by the sex of the animal. This supplemented the previous report by Barron and Tahmisian of enzymatic differences between male and female muscle homogenates. Accordingly, the present communication is concerned with the relative capabilities of the various cockroach tissues, male and female, to dephosphorylate ATP, and with the influence of bivalent cations on this process. From such findings, further investigations on the mechanism of nucleotide breakdown in insects can be logically approached.

## Experimental Procedures

*Preparation of tissues.* Adult male and female roaches were dissected in 0.9% KCl and the desired tissues were removed as described previously (Sacktor and Bodenstein, 1952). The tissues, immediately prior to enzymatic assay, were homogenized for 30 seconds in 1.0 ml. KCl solution with a Potter-Elvehjem homogenizer. Separate determinations were made on the tissues from each of 10 roaches. Because of the small amount of tissue available from one roach, the separate tissues of two roaches were pooled for the experiments with brain and Malpighian tubes. Thus, 20 roaches were used for the 10 determinations of the enzymatic activity of these tissues.

*Determination of enzyme activity.* The dephosphorylation of ATP was determined by assaying for inorganic phosphate after 15 minutes of incubation at room temperature (22 to 25 degrees C.). The final concentration of ingredients was: tris (hydroxymethyl) aminomethane buffer, pH 7.4, 0.03 *M*; ATP (Na salt from Pabst Laboratories) 0.2%; $MgCl_2$, or $CaCl_2$, $10^{-3}$ *M*; 0.2 ml. of tissue homogenate (except muscle, where but 0.1 ml. was used); and 0.9% KCl to a final volume of 1.0 ml. Inorganic phosphate was measured by the method of Sumner (1944) in a Klett-Summerson photoelectric colorimeter with a No. 66 filter. Zero time was at the instant of addition of enzyme, and the value determined at this time was subtracted from the final value. With this procedure for enzymatic assay, the rate of dephosphorylation of ATP is directly proportional to the amount of enzyme

present and is linear with time during the 15-minute incubation period. A representative set of data is depicted in Figure 1. Furthermore, no inorganic phosphate is liberated either in the absence of homogenate or in the presence of tissue but in the absence of ATP.

*Determination of protein.* Protein was determined by the method of Lowry *et al.* (1951). Due to the presence in the fat body of urates, which interfere with the color reaction, aliquots of this tissue homogenate were first extracted with

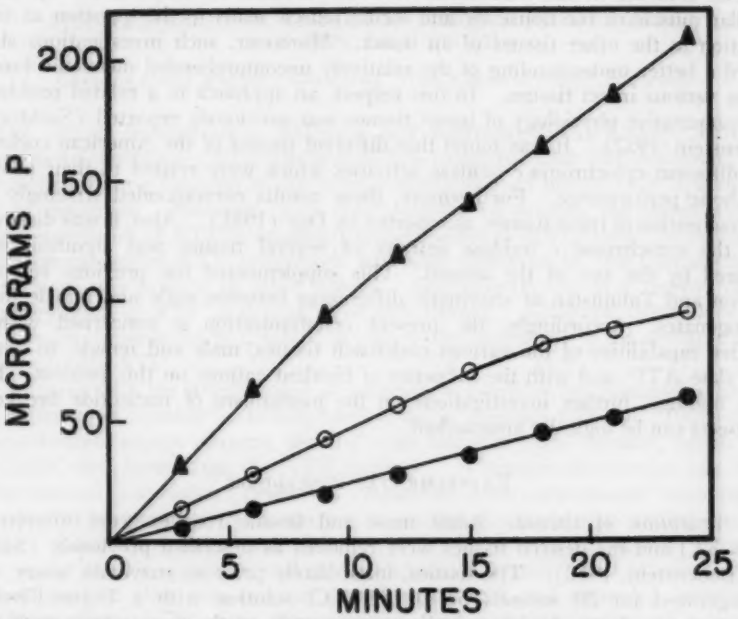



FIGURE 1. The effect of varying concentrations of muscle homogenate on the course of dephosphorylation of ATP. The following symbols: ▲, ○ and ● represent the liberation of inorganic phosphate in the presence of 0.2 ml., 0.1 ml. and 0.05 ml., respectively.

acetone and subsequently the protein was precipitated twice with trichloroacetic acid. Crystalline bovine serum albumin was used as the standard protein.

## RESULTS

The relative capabilities of various cockroach tissues, male and female, in dephosphorylating ATP, and the influence of bivalent cations on this reaction are shown in Tables I and II.

*Dephosphorylation by tissues.* The data in Tables I and II reveal that insect tissues differ in their ability to dephosphorylate ATP. In general, based on their activity, they can be grouped into three categories. These are (1) those with greatest activity: muscle, fat body and Malpighian tubes; (2) those with moderate

TABLE I

*Dephosphorylation of ATP*
*Males*

| Tissue | Control | Calcium | Magnesium |
|---|---|---|---|
| | micrograms P/hour/mg. protein | | |
| | Mean ± S.E. | Mean ± S.E | Mean ± S.E. |
| Muscle | 119 ± 11 | 237 ± 13 | 516 ± 39 |
| Midgut | 17 5 | 29 6 | 59 10 |
| Foregut | 33 6 | 59 11 | 77 8 |
| Hindgut | 25 5 | 44 8 | 103 14 |
| Malpighian tubes | 94 21 | 109 23 | 145 27 |
| Fat body | 140 33 | 179 44 | 353 38 |
| Brain | 60 10 | 67 24 | 113 16 |
| Nerve cord | 45 13 | 54 9 | 161 25 |

Control values represent micrograms P liberated in the absence of added bivalent ions. Ca and Mg ion concentration was $10^{-3}$ *M*. Each datum is the average of 10 determinations.

activity: brain and nerve cord; (3) those of low activity, namely, the three portions of the alimentary canal: hindgut, foregut and midgut. Muscle, as might have been predicted, is the most active. The rate of dephosphorylation by the fat body suggests that this organ may be the site of more extensive cellular metabolism than heretofore realized. This supports recent observations by Bodenstein (1953) on the metabolic role of the fat body. He found that the intermediate metabolism of this tissue responded considerably to changes in the hormonal situation. Although for the most part the gut portions possess relatively little dephosphorylating activity, a notable exception is the hindgut of the female roach.

TABLE II

*Dephosphorylation of ATP*
*Females*

| Tissue | Control | Calcium | Magnesium |
|---|---|---|---|
| | micrograms P/hour/mg. protein | | |
| | Mean ± S.E. | Mean ± S.E. | Mean ± S.E. |
| Muscle | 231 ± 31 | 396 ± 33 | 556 ± 71 |
| Midgut | 20 5 | 26 6 | 65 12 |
| Foregut | 45 10 | 68 13 | 99 22 |
| Hindgut | 99 18 | 111 21 | 148 27 |
| Malpighian tubes | 118 23 | 143 21 | 120 19 |
| Fat body | 95 26 | 127 42 | 159 54 |
| Brain | 47 10 | 64 10 | 99 17 |
| Nerve cord | 74 16 | 74 17 | 140 27 |

Control values represent micrograms P liberated in the absence of added bivalent ions. Ca and Mg ion concentration was $10^{-3}$ *M*. Each datum is the average of 10 determinations.

*Effect of bivalent ions.* The data in Tables I and II demonstrate the influence of bivalent cations on ATP dephosphorylation. It is apparent that both Ca and Mg ions stimulate the release of inorganic phosphate from ATP, but that $Mg^{++}$ is more effective. There are, however, differences in the magnitude of response to these ions with the different tissues. Thus, muscle is activated almost 100% by $Ca^{++}$ whereas the slight stimulation observed with brain, nerve cord, Malpighian tubes and fat body was not statistically significant. The Ca-activation noted with the gut parts may represent the effect of this cation on the intrinsic musculature. Mg ions do not have a considerable effect on dephosphorylation of ATP by the Malpighian tubes but, in contrast, do activate the other tissues notably.

*Effect of sex.* A comparison of Tables I and II indicates that the dephosphorylation of ATP by a given tissue may be influenced by the sex of the roach. Thus, the enzymatic activity of the muscle in the female is significantly (beyond the 0.01 level) greater than that in the male, either in the absence of bivalent cations or in the presence of Ca ions, but not in the presence of Mg ions. The failure to

TABLE III

*Inhibition of dephosphorylation of ATP by p-chloromercuribenzoate*

| Inhibitor conc. (*M*) | Per cent inhibition | | |
|---|---|---|---|
| | Control | Calcium | Magnesium |
| $5 \times 10^{-6}$ | 7 | 13 | 6 |
| $1 \times 10^{-5}$ | 40 | 28 | 31 |
| $5 \times 10^{-5}$ | 82 | 73 | 41 |
| $1 \times 10^{-4}$ | 89 | 85 | 54 |
| $5 \times 10^{-4}$ | 96 | 96 | 84 |
| $1 \times 10^{-3}$ | 97 | 97 | 92 |
| $5 \times 10^{-3}$ | 100 | 100 | 98 |
| $1 \times 10^{-2}$ | 100 | 100 | 100 |

Muscle of the male roach was used in these experiments. Control values represent the per cent inhibition in the absence of added bivalent ions. Ca and Mg ion concentration was $10^{-2}$ *M*.

observe a sex difference with muscle activated by Mg ions is in agreement with the results of Gilmour and Calaby, who found that the sex of the locust had no influence on the activity of the Mg-activated soluble apyrase. As in muscle, the hindgut of the female is more active than that of the male in the absence of bivalent cations or when stimulated by Ca ions. On the other hand, the fat body of the male, in the presence of Mg ions, is more active than that of the female. The differences, due to sex, exhibited by the other tissues are not statistically significant.

*Effect of inhibitors.* The effect of several known inhibitors of dephosphorylation on the activity of male roach muscle homogenates was ascertained since, in a previous study on the dephosphorylation of ATP by house fly flight muscle (Sacktor, 1953), it was found that azide and p-chloromercuribenzoate inhibited mitochondrial ATPase whereas fluoride did not. Fluoride, however, effected considerable inhibition of a soluble ATPase. In contrast with these observations on house fly flight muscle, it was found in the present study that $NaN_3$ and NaF at concentrations as high as $10^{-2}$ *M* are without appreciable effect on the dephos-

phorylation of ATP by roach muscle. However, p-chloromercuribenzoate inhibits dephosphorylation here also, as shown in Table III. A similar effect of this -SH inhibitor was noted by Gilmour and Calaby with their enzyme preparation from the locust.

A comparison of the present results with those obtained with the house fly (Sacktor, 1953) indicates that p-chloromercuribenzoate is approximately 100 times as effective an inhibitor in the roach. The data suggest further that inhibition in the roach is somewhat lessened in the presence of Mg ions. With rat liver mitochondrial ATPase, however, Novikoff *et al.* (1952) found no difference in the inhibition by p-chloromercuribenzoate ($5 \times 10^{-4}$ *M*) in the presence of Mg or Ca ions.

## Discussion

The data presented show that various cockroach tissues differ in their ability to dephosphorylate ATP and that both Ca and Mg ions activate dephosphorylation. The magnitude of stimulation by these cations depends on the particular tissue. It should be emphasized, however, that these results were obtained with the use of a standard experimental procedure for all tissues. This procedure, which is based on optimal conditions for muscle, need not represent the ideal situation for the other tissues.

Complete characterization of the dephosphorylating mechanisms with the various tissues must await further experimentation; nevertheless, an interesting comparison between the enzymatic activities found in roach muscle and in vertebrate muscle is now possible. In a recent study of the biochemical differences between red and white muscle, Lawrie (1952) found that red muscle had more myoglobin and a higher activity of cytochrome *c* oxidase, succinic dehydrogenase and succinoxidase than did white muscle, but, conversely, a lower ATPase activivty. The coloration of red muscle was attributed to the greater content of myoglobin. In the roach, however, the muscle of the male is reddish in color, whereas that of the female is much lighter, almost white (Sacktor and Bodenstein, 1952; Edwards, 1953). It was found, too, that these color variations were reflected in the cytochrome *c* oxidase (Sacktor and Bodenstein, 1952) as well as succinic-cytochrome *c* reductase activities (Sacktor, unpublished data); for, muscles with more coloration had a higher activity; and, as with vertebrate muscle, the dephosphorylation of ATP in female (white) muscle was greater. Furthermore, Barron and Tahmisian (1948) reported more cytochrome *c* and Fe in the male roach muscle. Contrary to the situation in vertebrates, there is no evidence for the presence of myoglobin in roach muscle. These facts indicate that in insects, at least, the color differences may be due to differences in the cytochrome content, and suggest that cytochromes as well as myoglobin are possibly concerned in the color of vertebrate muscle.

Despite many similarities between insect and vertebrate muscle, distinctions can be made even between the various insect species. Differences in the mechanism of ATP breakdown by house fly flight muscle and locust muscle have been described above. In addition, dissimilarities in the action of several inhibitors of dephosphorylation were found between roach muscle homogenates and house fly flight muscle preparations. In the roach muscle, p-chloromercuribenzoate inhibited dephosphorylation whereas azide and fluoride did not. In contrast, with mitochon-

dria isolated from the specialized flight muscles of the fly, ATPase was inhibited by azide as well as by p-chloromercuribenzoate (Sacktor, 1953). Furthermore, fluoride prevented orthophosphate liberation by a soluble fraction obtained from these specialized fibrillar muscles. Other species-specific enzymatic characteristics have been noted recently in other insects. Metcalf and March (1949) found that certain organic phosphates inhibited fly brain cholinesterase but had little effect on bee brain cholinesterase. Supplemental evidence on species variations was obtained by Rockstein and Levine (1951) in their studies on acid phosphatases in five insect species. From these numerous heterogeneities, it is becoming increasingly evident that generalizations on the enzymatic properties of all insects are no longer valid. Careful examination of such parallels and differences in insects may well yield fruitful leads for investigation in the tissues of other animals.

## Summary

1. The dephosphorylation of ATP by various tissues of the American cockroach was investigated. It was found that these tissues differ in their dephosphorylating activity and may be rated, in decreasing order, approximately as follows: muscle, fat body, Malpighian tubes, nerve cord, brain, hindgut, foregut and midgut.

2. In general, both Mg and Ca ions activated the breakdown of ATP, although Mg was more effective. The magnitude of activation by these bivalent cations depended on the tissue.

3. Some differences due to sex were found. The dephosphorylating activity of the muscle and hindgut of the female was significantly greater than that in the male either in the absence of bivalent cations or in the presence of Ca ions. The fat body of the male, in the presence of Mg ions, was more active than that of the female.

4. The dephosphorylation of ATP by roach muscle was inhibited by p-chloromercuribenzoate, but not by azide and fluoride. Since these three compounds were effective inhibitors of house fly flight muscle ATPase, this distinction further emphasizes the heterogeneity of ATP breakdown mechanisms in insects.

## LITERATURE CITED

Albaum, H., and M. Kletzkin, 1948. Adenosine triphosphate from *Drosophila melanogaster*. *Arch. Biochem.*, **16**: 333–337.

Barron, E. S. G., and T. N. Tahmisian, 1948. The metabolism of cockroach muscle (*Periplaneta americana*). *J. Cell. Comp. Physiol.*, **32**: 57–76.

Bodenstein, D., 1953. Studies on the humoral mechanisms in growth and metamorphosis of the cockroach, *Periplaneta americana*. III. Humoral effects on metabolism. *J. Exp. Zool.* (In press.)

Calaby, J. H., 1951. Adenosine triphosphate from insect muscle. *Arch. Biochem. Biophys.*, **31**: 294–299.

Chin, C. T., 1951. High temperature coefficient of enzymic activity of the muscle of the American cockroach (*Periplaneta americana*). *Arch. Biochem. Biophys.*, **31**: 333–335.

Day, M. F., 1951. Studies on the digestion of wool by insects. III. A comparison between the trachea of the midgut of *Tineola* larvae and that of other insect tissues. *Austral. J. Scientific Res.*, **B4**: 64–74.

Edwards, G. A., 1953. *In:* Insect physiology. Edited by K. D. Roeder. John Wiley and Sons, Inc., New York: 96–146.

Gilmour, D., 1948. Myosin and adenylpyrophosphatase in insect muscle. *J. Biol. Chem.*, **175**: 477–478.

GILMOUR, D., AND J. H. CALABY, 1952. The magnesium-activated apyrase of insect muscle. *Arch. Biochem. Biophys.*, **41**: 83–103.

LAWRIE, R. A., 1952. Biochemical differences in red and white muscle. *Nature*, **170**: 122–123.

LOWRY, C. H., N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275.

METCALF, R. L., AND R. B. MARCH, 1949. Studies on the mode of action of parathion and its derivatives and their toxicity to insects. *J. Econ. Entom.*, **42**: 721–728.

NOVIKOFF, A. S., L. HECHT, E. PODBER AND J. RYAN, 1952. Phosphatases of rat liver. I. The dephosphorylation of adenosine triphosphate. *J. Biol. Chem.*, **194**: 153–170.

ROCKSTEIN, M., AND L. LEVINE, 1951. Enzymes in insects. Acid phosphatase. *Ann. Entom. Soc. Amer.*, **44**: 469–472.

SACKTOR, B., 1953. Investigations on the mitochondria of the house fly (*Musca domestica*). I. Adenosine triphosphatase. *J. Gen. Physiol.*, **36**: 371–387.

SACKTOR, B., AND D. BODENSTEIN, 1952. Cytochrome *c* oxidase activity of various tissues of the American cockroach, (*Periplaneta americana* L.). *J. Cell. Comp. Physiol.*, **40**: 157–160.

SUMNER, J. B., 1944. Method of colorimetric determination of P. *Science*, **100**: 413–414.

# PHYSIOLOGY OF INSECT DIAPAUSE. V. ASSAY OF THE GROWTH AND DIFFERENTIATION HORMONE OF LEPIDOPTERA BY THE METHOD OF TISSUE CULTURE

EDMOND L. SCHMIDT [1] AND CARROLL M. WILLIAMS [2]

*The Biological Laboratories, Harvard University, Cambridge 38, Mass.*

The metamorphosis of insects, like the growth and maturation of vertebrates, is under the control of an endocrine *system*. In the Lepidoptera, the Diptera, the Hemiptera, and the Blattoidea, this system is known to include a minimum of three endocrine tissues—the brain, the prothoracic glands, and the corpora allata. The *brain hormone* is the secretory product of certain highly specialized neurones in the cerebral ganglion; its principal target appears to be the prothoracic glands. Under the tropic stimulation of the brain hormone the prothoracic glands secrete the *growth and differentiation hormone*. And it is apparently this prothoracic gland hormone which reacts with the tissues to promote growth, molting, and metamorphosis (Williams, 1952a). Within the immature insect, there is yet a third hormone whose source is the corpora allata. The corpus allatum hormone is a conservative factor—a *status quo hormone*—which inhibits metamorphosis by modifying the response of the immature tissues to the growth and differentiation hormone (Williams, 1952b).

Though all these conclusions are based on substantial evidence, it is worth recalling that neither the brain hormone, nor the growth and differentiation hormone, nor the *status quo* hormone has ever been demonstrated by any method of chemical or *in vitro* assay. Consequently, it has been necessary to judge the hormonal action by recourse to such indirect methods as the extirpation and implantation of living endocrine organs, parabiosis, and similar procedures. Indeed, none of the above-mentioned hormonal effects is duplicated when one removes blood from an insect and injects it into a test animal. This fact is scarcely mentioned in the literature (Schürfeld, 1935; Plagge, 1938), though it is a reasonable presumption that such experiments have been carried out by most investigators. In studies of the Cecropia silkworm, we have failed in repeated attempts to demonstrate endocrine activity by the injection of blood. Thus, in numerous experiments performed on brainless diapausing pupae, two-thirds of the blood was drained and replaced by that of mature larvae or developing adults. We have continued this procedure on individual pupae at weekly intervals from one to seven times. In no instance did the transfused blood have any detectable effects on the test animal.

[1] Former Predoctoral Fellow of the Lalor Foundation. Present address: Department of Zoology, University of Connecticut, Storrs, Connecticut.

[2] This study was aided by the Lalor Foundation, by a research grant from the U. S. Public Health Service, and by an Institutional Grant to Harvard University from the American Cancer Society, Inc.

Experiments of this type have been the principal basis for the view that the hormones of insects are conveyed in the tissues rather than in the blood. If the blood has no demonstrable endocrine activity, then perhaps the hormones of insects, like the auxins of plants (Thimann, 1952), may be transported from cell to cell rather than in the "sap."

With the exception of the blood-borne factor which promotes puparium formation in the higher Diptera (Fraenkel, 1935), the only substantial evidence that hormones concerned with growth and metamorphosis are present in the blood has hitherto been derived from parabiosis experiments. Thus, when two insects are grafted together so that they share the same blood, the one can supply hormones to the other. However, under further analysis, the two animals are found to grow together and attain organic continuity before the hormonal effect is manifested (Bodenstein, 1938; Williams, 1946). Moreover, this tissue union is established even when glass or plastic tubes have been interposed between the animals (Williams, 1947; Lüscher, 1948). The parabiosis experiments have therefore intensified rather than resolved the problem of the mode of transport of insect hormones.

The central and persistent difficulty has been the lack of any practical method of assaying the insect hormones. In the absence of such tests, it has been impossible to detect the hormones in the blood or in the tissues. It has also been impossible to proceed with their extraction, purification, and chemical characterization.

The present study was directed toward the development of an assay for the growth and differentiation hormone. For reasons considered above, it was obvious that the intact insect failed to provide a basis for such a test. Attention was therefore focussed on the culture of an individual tissue in the hope that an *in vitro* system might furnish a method of biological assay.

Practical considerations directed the choice of an appropriate tissue. The latter should be readily available and in a uniform state at the outset. It should undergo prompt response at the beginning of adult development; *i.e.,* during the period when the action of the growth and differentiation hormone is uncomplicated by the simultaneous presence of the *status quo* hormone. Moreover, its response to the growth and differentiation hormone should be accompanied by conspicuous change. Finally, the tissue should be simple to culture.

One tissue above all others appeared to satisfy these requirements; namely, the male sex cells of the diapausing pupal testes—a cell type which at the outset of adult development undergoes prompt and spectacular transformation into spermatozoa. Could such cells be cultured *in vitro?* Assistance in so doing was sought in a survey of the scattered literature.

## In Vitro Culture of Insect Tissues

The first attempt to culture insect tissues was that of Goldschmidt (1915, 1916, 1917). Working in Harrison's laboratory, Goldschmidt cultured the male sex cells of Cecropia pupae and observed definite maturation in simple hanging-drop preparations. Unfortunately, no record was made of the exact status of the "pupae" that served as donors of blood; consequently, no endocrinological significance was apparent in Goldschmidt's results.

Similar hanging-drop preparations have subsequently been used to observe meiosis in the male germ cells of adult Orthoptera and Hemiptera (Lewis and

Robertson, 1916; Bêlâr, 1929; Baumgartner and Payne, 1930; Ris, 1949). But in all these instances the germ cells were observed for only a few hours after their isolation, and the development *in vitro* was clearly a continuation of processes already underway in the intact insect.

The only direct support for the view that the *in vitro* growth of insect tissues may depend on hormonal factors is an isolated study reported twenty-five years ago. In experiments designed to solve a morphological problem, Frew (1928) observed that the leg discs of blowfly larvae evaginated and underwent definite development in a culture medium prepared from early pupae, but failed to do so in a medium prepared from older pupae or from larvae. However, Frew's experiments are difficult to interpret since the data are presented in meager detail and technical difficulties are emphasized.

Numerous observations have been made of the behavior of cultured blood cells. In one of the early studies in this field, Glaser (1917) found that the blood cells of caterpillars and adult grasshoppers underwent limited multiplication in simple cultures, either in blood or in Locke's solution. However, several other investigators have been unable to detect mitotic activity in hanging-drop preparations of blood. Goldschmidt (1916) observed only amitotic activity as the blood cells of Cecropia formed a syncytial tissue in the cultures. Similar *in vitro* formations of blood cell syncytia have been observed in *Oryctes* (Lazarenko, 1925), *Periplaneta* (Taylor, 1935; Trager, 1935), larval *Bombyx mori*, and pupal *Platysamia cecropia* (Trager, 1935). Fischer and Gottschewski (1939) report the multiplication of *Drosophila* blood cells in an artificial medium which is not described in detail; growth was also observed in isolated leg and wing anlagen.

But the continuous multiplication of cells, comparable to that obtained in cultures of vertebrate tissues, has apparently not been achieved on insect material. The nearest approach was Trager's (1935) finding that certain cells in the ovary of mature silkworm larvae underwent considerable proliferation when cultured in a medium containing a small quantity of larval blood. Moreover, Charin (1930) reports the migration and limited multiplication of certain lepidopterous tissues *in vitro*—a finding reminiscent of the so-called "residual growth" of vertebrate tissues in the absence of embryonic extract. In the remaining instances in which insect tissues have been successfully explanted (Murray, 1926; Gottschewski and Fischer, 1939; Stern, 1940), residual activity was apparent, but no actual growth occurred.

From this survey of the literature, it is apparent that the cultivation of insect tissues has not been a widely used or highly successful technique. In view of the fact that morphogenetic activity in the insect itself is intermittent and evidently related to growth-controlling hormones, the scanty success achieved in the culture of insect tissues suggests that these same endocrine factors may be required for the growth of isolated tissues.

## Development of Sex Cells in the Intact Insect

The testes of the Cecropia silkworm, at all stages, are divided internally into four tubules by three septa radiating from the region of the vas deferens. The tubules contain the germ cells, the majority of which are gathered into small balls and enclosed by an envelope of flattened cells—the whole forming the so-called "germinal cysts" (Dederer, 1907; Omura, 1936). The latter are either free in the fluid which

fills the testicular tubules or suspended in grape-like clusters from minute tracheoles. The maturity of the germ cells can be judged by their size, by the number in each cyst, and by the fact that their early development transforms the cysts into hollow spheres.

During the prepupal period the cells in a few of the more mature cysts undergo one or both maturation divisions, all of the cells in a given cyst dividing simultaneously. In a small proportion of cysts, spermatids begin to differentiate at this time—a process invariably accompanied by considerable elongation of the cyst as a whole. Cells which undergo this precocious maturation during the prepupal period appear to die shortly thereafter. Thus the testes of diapausing pupae include cysts containing degenerate cells with pycnotic nuclei, but no recognizeable spermatids. Consequently, the germ cells in the pupal testes closely resemble those present in mature larvae and range from spermatogonia to primary spermatocytes.

During the pupal diapause there is no indication of further development in any of these cells. However, immediately after the termination of diapause and the initiation of adult development, a rapid and impressive maturation takes place. Mitotic divisions occur in the immature cysts containing spermatogonia, while meiotic divisions occur in the more mature cysts containing primary spermatocytes. These changes are synchronized with the first signs of adult development in the pupal hypodermis. Shortly thereafter, even the immature germ cells, comparable to those commonly observed in larvae midway the fifth instar, promptly transform into spermatids. By the end of the first week of adult development, nearly all the sex cells in the testes have been converted to spermatids.

## Materials and Methods

Sex cells from the testes of diapausing pupae of *Platysamia cecropia* and *Samia walkeri* (*cynthia*) were cultured in the blood of larvae, diapausing pupae, developing adults, and adults of the same species. The cultures were prepared as follows:

A male diapausing pupa was selected and surface-sterilized by immersion in a solution of 0.05% mercuric chloride in 50% ethyl alcohol, followed by thorough rinsing in sterile distilled water—a treatment that had no detectable effects on the insects at any stage in development. A transverse slit was then cut in the dorsal region between the fourth and fifth abdominal segments and, with slight pressure on the abdomen, the testes were caused to herniate. Each testis was then transferred to a depression slide containing blood previously collected from a suitable donor.

Rupture of the testicular envelope liberated thousands of germinal cysts into the small pool of blood. By means of a fine pipette a drop of the resulting suspension was placed on the underside of a coverslip and the latter sealed to a depression slide with melted paraffin. It was impossible to pipette a uniform suspension of cysts, and the number included in each culture varied considerably. Ordinarily, each preparation contained at least 75 cysts—indeed, a relatively large number seemed to favor the survival of the culture. Five or six cultures were usually prepared from each sample of blood. Each culture was immediately examined under the compound microscope for any indication of development; it was then placed at the constant temperature of 25° C. or 30° C. and examined at daily intervals.

The blood used as the culture medium was obtained by slicing the cuticle and underlying hypodermis from the facial region of donor insects and draining the

blood into a depression slide containing a few small crystals of phenylthiourea (twice recrystallized from the Eastman product). The blood of these silkworms, like that of most insects, undergoes rapid darkening unless the activity of tyrosinase is inhibited. This darkening results from the enzymatic oxidation of phenols to quinones. Such darkened blood is wholly unsuitable as a culture medium and, in fact, is poisonous. Phenylthiourea specifically inhibits tyrosinase and opposes these changes without, in itself, being toxic. It was the use of this chemical that made the present experiments technically possible.

Blood from developing adults contains a large amount of cellular debris from disintegrating fat body. This was largely eliminated by allowing the particulate matter to settle and withdrawing the relatively clear blood with a pipette. In a number of experiments, the blood was centrifuged to remove all cells—a procedure which seemed to be inconsequential.

The preparation of cultures was accomplished with sterile instruments and glassware; the technique was simple and sufficiently rapid to make elaborate precautions against air-borne organisms unnecessary. Approximately 2% of the cultures eventually showed contamination with microorganisms. Since a number of identical cultures were always prepared, contamination was not a major source of difficulty.

## Pupal Germ Cells Cultured in Blood of Pupae, Developing Adults, and Adults

The results of 179 successful cultures prepared in the blood of post-larval donors are summarized in the lower portion of Table I. This total includes all cultures in which any development was detected, though in some instances the cultures survived a relatively short time. Since the onset of development was occasionally delayed until the fourth day, the absence of development was not considered significant until the fourth day; cultures showing no development but failing to survive four days are not included in the data. The death of germ cells *in vitro* seemed to be closely followed or even preceded by visible degenerative changes, and it has been assumed that the cells were alive until these changes appeared.

### *1. In blood of diapausing and previously chilled pupae*

As indicated in Table I, germ cells in the majority of cultures prepared in the blood of pupae which had been in the dormant state for 5 to 16 weeks retained their original configuration (Fig. 1A) and showed no development whatsoever, notwithstanding the fact that many of the cultures survived for a week or longer. In certain preparations the meiotic divisions were evident in an occasional cyst (Fig. 1B). However, the cells in such cysts underwent degeneration within two or three days without forming elongated spermatids, and development was initiated in no additional cysts. On rare occasions, cysts like those shown in Figures 1C and 1D were detected in cultures several days old. Cultures showing any of these patterns of minimal response have been listed as undergoing "slight development."

Results identical to those obtained in the blood of dormant pupae were observed in cultures prepared in the blood of previously chilled pupae—that is, pupae which had been stored at 5° C. for a sufficient period to assure the initiation of development after about two weeks' exposure to 25° C. Altogether it is evident that germ

TABLE I

*Pupal germ cells cultured in blood from larvae, prepupae, pupae, and developing adults*

| Source of blood | Age of donors (days) | No. of donors | No. of cultures | Development in cultures | | |
|---|---|---|---|---|---|---|
| | | | | None | Slight | Progressive |
| Mature 5th instar larvae, not yet spinning | 0 | 3 | 15 | 14 | 1 | 0 |
| Larvae, spinning outer capsule of cocoon | 0–1 | 3 | 10 | 8 | 2* | 0 |
| Larvae, spinning inner capsule of cocoon | 1–2 | 5 | 34 | 5 | 0 | 29 |
| Larvae, finished spinning cocoon | 2–5 | 3 | 9 | 0 | 3* | 6 |
| Green prepupae | 5–8 | 3 | 11 | 0 | 1* | 10 |
| White prepupae | 8–9 | 2 | 10 | 0 | 4* | 6 |
| Fresh pupae | 9–10 | 2 | 13 | 0 | 2* | 11 |
| Diapausing pupae | After 3 weeks at 25° C. | 4 | 17 | 2 | 9 | 6 |
| Diapausing pupae | After 5 to 16 weeks at 25° C. | 13 | 40 | 26 | 14** | 0 |
| Chilled diapausing pupae | After 12 to 35 weeks at 5° C. | 7 | 23 | 20 | 3* | 0 |
| Developing adults | 1st to 5th day | 15 | 47 | 0 | 6* | 41 |
| Developing adults | 7th to 18th day | 6 | 18 | 0 | 2* | 16 |

* All cultures died within 48 hours after development was initiated.
** Six cultures died within 48 hours after development was initiated.

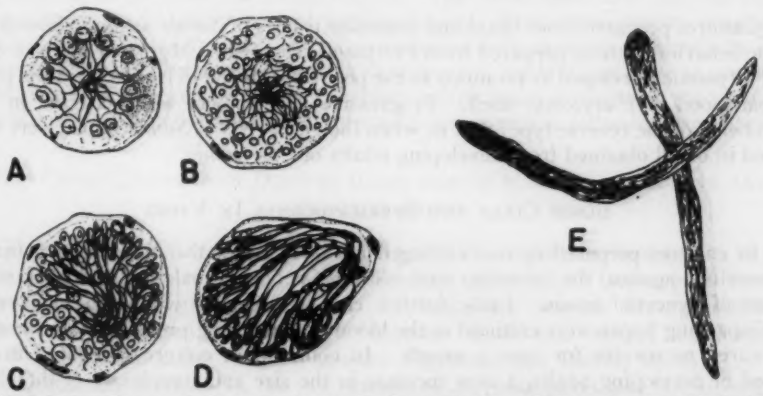



FIGURE 1. Characteristic stages in the development of pupal Cecropia sex cells in hanging-drop cultures of active blood. *A* is an optical section of a testicular cyst from a diapausing pupa; the enclosed cells are primary spermatocytes. *B* shows a typical cyst after several days of culture; the sex cells have just completed meiosis. Development proceeds in *C* to yield early spermatids within the still spherical cyst. In *D* the differentiation of spermatids has continued and the cyst as a whole has begun to elongate. *E* records the final stage of *in vitro* differentiation; the cysts are greatly elongated and contain well-differentiated spermatids. *A* through *D*, × 250; *E*, × 140.

cells are incapable of undergoing rapid or extensive development in the blood of diapausing or previously chilled pupae.

*2. In blood of developing adults and adults*

With the termination of pupal diapause and the initiation of adult development, a remarkable change was evident in the blood's capacity to promote the development of sex cells. Within 24 to 48 hours after cultures were prepared in the blood of developing adults, the cells in one or more cysts ordinarily showed the completion of the maturation divisions. Within the following 24 hours, meiosis usually occurred in many additional cysts. Meanwhile, the more precocious cysts underwent elongation, the nuclei moving to one pole and the axial filaments of the developing spermatids growing towards the other. At this stage a large proportion of the cysts corresponded to those diagrammed in Figures 1B to 1D. After a week in culture, the majority of cysts showed definite elongation; many were ten to fifteen times longer than wide and contained well differentiated spermatids (Fig. 1E).

Since the germ cells usually failed to survive longer than a week in culture and not infrequently died sooner, the final degree of differentiation was less impressive in those cultures where development was delayed or proceeded slowly. Characteristically, however, continuous progress was made toward the spermatid stage as long as the germ cells survived. Accordingly, "progressive development" was distinguished, not so much by the absolute amount of differentiation, as by its sustained progress. The results in Table I show that progressive development occurred in blood obtained from animals after the initiation of adult development.

*3. Germ cells of Platysamia cecropia cultured in the blood of Samia walkeri*

Cultures prepared from blood and testicular tissues of *Samia walkeri* showed the same behavior as those prepared from *Platysamia cecropia*. Moreover, the sex cells of *Platysamia* developed as promptly in the post-pupal blood of *Samia* as in the post-pupal blood of *Platysamia* itself. Progressive development also occurred in experiments of the reverse type; that is, when the germ cells of *Samia* pupae were cultured in blood obtained from developing adults of *Platysamia*.

## Blood Cells and Spermatogonia In Vitro

In cultures prepared in uncentrifuged blood, many of the blood cells flattened themselves against the coverslip and within two days coalesced to form small areas of syncytial tissue. Little further change occurred when the blood cells of diapausing pupae were cultured in the blood of diapausing pupae, though the cells appeared to survive for over a month. In contrast, in cultures prepared in the blood of developing adults, a slow increase in the size and complexity of the blood cell syncytia became evident after five or six days. In cultures maintained for two weeks or longer and subsequently fixed and stained, scattered mitotic figures were evident in the flattened blood cells.

In cultures prepared in the blood of early developing adults and maintained for longer than the usual period of one week, masses of small cells slowly appeared. These cells failed to correspond to any of the various types of blood cells, nor did

they show the latter's tendency to flatten and form syncytial tissues. Inspection of fixed and stained cultures revealed frequent mitotic figures.

Pupal testes invariably contain a small number of cells not yet enclosed within cysts. These cells, which are presumed to correspond to early spermatogonia, are inevitably released along with the cysts of late spermatogonia and spermatocytes when the testes are ruptured in the preparation of the cultures. The cells observed to multiply in cultures closely resembled the early spermatogonial cells in terms of the presence of a scanty, dense cytoplasm and a large vesicular nucleus.

It thus appears that the property of the blood of developing adults which promotes the differentiation of spermatocytes also stimulates cells of a different character, such as blood cells and spermatogonia, to divide. Consequently, it can truly be said that the active agent in the blood of developing adults promotes both growth and differentiation.

### Pupal Sex Cells Cultured in Larval Blood

Observations were made of the differentiation of pupal cells when cultured in larval blood. The preparation of such cultures was at first complicated by a jelly-like clotting of the blood when withdrawn from caterpillars. Fortunately, this viscous change, due apparently to adhesions between blood cells, disappeared when the blood was stored for 12 to 24 hours at 3° C. Under this circumstance, the cells formed dense clumps and the clear fluid residue could once more be used in culturing the pupal spermatocytes.

The results of cultures of pupal cysts in larval blood (Table I) indicate that the blood of mature fifth instar larvae is essentially inactive until a stage signalled by the spinning of the inner coat of the cocoon. The larval blood then becomes active in promoting the transformation of pupal sex cells. From these results, it appears that a factor required for the maturation of pupal cells increases markedly in concentration, not only at the outset of adult development, but also just prior to pupation. This factor, as indicated in Table I, persists in the blood throughout the prepupal period and then slowly falls to sub-threshold concentration following pupation.

### Sex Cells Cultured in Diluted Blood and in Mixtures of Active and Inactive Blood

The failure of the blood of diapausing pupae to support the maturation of sex cells might be attributed either to the presence of an inhibitory factor, or to the absence of a necessary growth hormone. To distinguish between these possibilities, cultures were prepared in mixtures of "dormant" blood and "active" blood and in blood diluted with insect Ringer's solution (Ephrussi and Beadle, 1936).

As recorded in Table II, the low activity of dormant blood was not altered by the addition of an equal volume of Ringer's solution. Hence, if the diapausing blood contains an inhibitory substance, the latter is not rendered sub-threshold by this degree of dilution. Table II also shows that active blood remained active after the addition of an equal volume of dormant blood. These findings indicate that the blood of developing adults contains a growth-promoting hormone which is in far lower titer in the blood of mature diapausing pupae.

TABLE II

*Pupal germ cells cultured in mixtures of bloods and in bloods diluted with Ringer's solution*

| Culture medium | Number of experiments | Number of cultures | Development in cultures | | |
|---|---|---|---|---|---|
| | | | None | Slight | Progressive |
| Blood from dormant pupae plus Ringer's solution; equal parts | 4 | 12 | 8 | 4* | 0 |
| Blood from developing adults plus Ringer's solution; equal parts | 5 | 17 | 0 | 0 | 17 |
| Blood from developing adults and dormant pupae; equal parts | 5 | 22 | 0 | 12** | 10 |

* All cultures died within 48 hours after development was initiated.
** Seven cultures died within 48 hours.

PROPERTIES OF THE GROWTH-PROMOTING SUBSTANCE PRESENT IN ACTIVE BLOOD

The nature and properties of the growth-promoting hormone in the blood of pupating larvae and developing adults were studied by subjecting active blood to various treatments and then re-testing its capacity to promote development *in vitro*.

*1. Effects of storage at 3° C. and of freezing*

Hormonal activity was retained when active blood was stored under refrigeration. As previously mentioned, blood collected from pupating larvae was routinely stored at 3° C. for 12 to 24 hours without loss of activity. Indeed, in two experiments involving a total of eight cultures, progressive development was observed in prepupal blood previously stored at 3° C. for four days. In another experiment, blood obtained from developing adults was frozen at − 29° C., then thawed at room temperature, then re-frozen and re-thawed. When tested in six cultures, full activity was observed—a finding which demonstrates that the hormone is stable at low temperatures.

*2. Effects of heat treatment*

Abrupt heating to temperatures above 60° C. caused the blood to coagulate in a solid mass. This difficulty was avoided by raising the temperature in steps of approximately 5°, and removing the precipitate by centrifugation after each step. By this procedure a small fraction of clear supernatant could be obtained even after heating the blood to 100° C.

When active blood was heated to 56° C., a scanty white precipitate formed; after centrifugation the supernatant proved suitable for use in the cultures. But when the temperature was raised to 60° C., a copious white precipitate appeared; the supernatant, when tested, now caused extreme fragmentation and granulation of the sex cells and death within a few hours—a result which we attribute to the altered osmotic pressure of the blood. Consequently, in order to test blood previously heated to temperatures above 60° C., it was necessary to mix the supernatant with an equal volume of untreated, inactive blood obtained from diapausing pupae.

When prepared in this manner, the culture medium regained its compatibility with the germ cells. In each such experiment the inactivity of the unheated diapausing blood was verified in control cultures.

Experiments testing the growth of spermatocytes in initially active blood are summarized in Table III. Pupal germ cells showed prompt and progressive development to spermatids when cultured in blood previously heated for fifteen minutes at 75° C. or at any lower temperature. In contrast, the behavior of cultures prepared with blood heated for ten minutes at 80° C. was indicative of a definite decrease in growth-promoting activity. Though three of four such cultures showed slight development after three days, no further development occurred, notwithstanding the fact that the cells survived for approximately seven days. The fourth culture likewise appeared to survive for about a week, but showed no development whatsoever. A similar negative result was observed in blood previously heated to 100° C. for ten minutes, though in three cultures the cells survived for at least six days. In

TABLE III

*Pupal germ cells cultured in media containing active blood previously heated to various temperatures*

| Preliminary heat treatment | | Number of satisfactory cultures | Development in cultures |
|---|---|---|---|
| Temp. (° C.) | Time (min.) | | |
| 56 | 25 | 10 | Progressive |
| 65* | 15 | 7 | Progressive |
| 70* | 15 | 6 | Progressive |
| 75* | 15 | 5 | Progressive |
| 80* | 10 | 4 | Slight |
| 100* | 10 | 3 | None |

* Supernatant diluted with equal parts of inactive blood.

summary, these results indicate that the blood's hormone is stable and unprecipitated by brief exposure to a temperature of 75° C., but is progressively destroyed or precipitated at higher temperatures.

### 3. *Effect of dialysis*

Approximately one ml. of active blood was placed in a viscous casing and dialyzed for 24 hours at 1° C. against 100 ml. of insect Ringer's solution. The undialyzed fraction, mixed with an equal volume of blood from dormant pupae, was tested in five cultures and found to retain its capacity to promote the development of pupal germ cells. The experiment was repeated, increasing the time of dialysis to two days. The undialyzed fraction, mixed with an equal volume of dormant blood, caused prompt development when tested. Evidently, the hormone is either a large molecule or is closely bound to such a non-dialyzable component.

## DISCUSSION

The tissue culture studies demonstrate that the blood of the Cecropia silkworm contains a growth factor whose concentration or activity undergoes systematic

change during the larval-pupal-adult transformation. Its initial low titer in the blood of the mature larva increases markedly just prior to the prepupal stage; *i.e.*, during the period when the growth and differentiation hormone induces the larva to pupate (Williams, 1952a). Within the newly formed pupa the factor then persists in gradually diminishing titer, only a trace being encountered in the blood of the diapausing pupa several weeks later.

For months thereafter the blood of the diapausing pupa remains virtually inactive. But just prior to the termination of diapause and during the ensuing first week of adult development the factor reappears in high titer—a change which correlates precisely with the period of action of the prothoracic glands (Williams, 1952a). For these several reasons we are persuaded that the factor which promotes the *in vitro* development of the sex cells is the same growth and differentiation hormone which, within the intact insect, is required for the growth and maturation of the gonads and of all other tissues (Williams, 1952b).

Efforts were made to demonstrate this fact by more direct procedures; namely, by the implantation of active prothoracic glands into cultures prepared in inactive blood. Experiments of this type were complicated by bacterial contamination of the fragments of the prothoracic glands during their dissection from developing adults. Eventually, three hanging-drop cultures were prepared containing both germinal cysts and fragments of living prothoracic glands. Though the sex cells and the glandular cells appeared to survive for several days, no development took place. Similar negative results were obtained in cultures fortified with extracts of active prothoracic glands in saline or in inactive blood.

The apparent failure of the prothoracic glands to continue their secretory activity under the highly artificial conditions existing in hanging-drop cultures is not surprising, particularly in view of Fukuda's (1944) finding that a normal oxygen supply is necessary for the secretory activity of the prothoracic glands—a condition obviously not met in small, sealed cultures.

If one accepts the tissue culture test as a valid assay for the growth and differentiation hormone, then it is clear that the isolated testicular tissue is far more sensitive to the hormone than are the intact testes or the insect as a whole. The isolated germ cells respond to concentrations of growth and differentiation hormone that are sub-threshold within the intact insect. Thus the sex cells developed promptly when cultured in the blood of recently pupated animals in which all development had ceased (Table I). Indeed, the blood of pupae which had been dormant for months not infrequently caused a slight but definite response of the cultured cells. Moreover, it is worth recalling that during the second week of adult development the blood showed considerable hormonal activity, notwithstanding the fact that the prothoracic glands, the source of the hormone, are known to undergo complete degeneration during the first week of adult development. While attesting to the stability of the growth and differentiation hormone within the insect, this finding suggests the further possibility that the hormone may continue to influence the development of the insect during periods when the prothoracic glands are inactive or even absent, as, for example, during the maturation of the eggs.

There is clear evidence that one reason for the high sensitivity of the *in vitro* procedure is the absence of the testicular envelopes which are interposed between the sex cells and the blood within the intact insect. During the period of prepupal

development this barrier apparently shields the germ cells from the threshold concentration of hormone in the surrounding blood. For if the sex cells are removed from the testes of prepupae and cultured in the blood of the very same animals, normal spermatogenesis is observed. And, as we have seen, the same is true within the newly formed pupa where the concentration of hormone is sufficient to cause spermatogenesis *in vitro* but not *in vivo*.

The sensitive response of cultured germ cells affords the first direct evidence that the growth and differentiation hormone of insects is, indeed, a blood-borne hormone; it also provides a means of recognizing this factor. The fact that the germ cells of either *Platysamia cecropia* or *Samia walkeri* develop in the blood of the opposite species demonstrates that the growth and differentiation hormone of Lepidoptera is, to this degree, neither species- nor genus-specific. The hormone also appears to be non-dialyzable and destroyed or precipitated by exposure to temperatures above 75° C.—properties which suggest that the growth and differentiation hormone is either a protein or a smaller molecule tightly conjugated to a protein. Though the tissue culture technique is only roughly quantitative in its present form, the growth and differentiation hormone, according to the evidence already at hand, appears to be relatively stable within the insect and to wax and wane in synchrony with the recurrent bouts of secretory activity by the prothoracic glands.

Dietrich Bodenstein, Dr. Leigh E. Chadwick, Dr. Berta Scharrer, and Prof. Ernst Scharrer were most helpful in reading this paper in manuscript form.

## Summary

1. Spermatogonia and spermatocytes isolated from the testes of dormant pupae of the silkworms, *Platysamia cecropia* and *Samia walkeri* (*cynthia*), promptly develop into well differentiated spermatids when cultured in hanging-drops of blood obtained from pupating larvae or developing adults of these species. Comparable development does not occur in cultures prepared in blood obtained from diapausing pupae or from mature larvae prior to a well-defined period before the onset of the prepupal stage.

2. The *in vitro* development of the sex cells signals the presence of a growth-promoting hormone. In inactive blood, as during the pupal diapause, this hormone is ordinarily in sub-threshold titer.

3. In addition to promoting the maturation of spermatocytes, the hormone causes the *in vitro* multiplication of blood cells and spermatogonia.

4. Pupal blood is active in promoting the development of germ cells *in vitro* after the initiation of adult development; the blood of mature larvae becomes active at a stage signalled by the spinning of the inner coat of the cocoon. This timing corresponds precisely with the periods during which the prothoracic glands secrete the growth and differentiation hormone which provokes the pupation of the larvae and the adult development of the pupa.

5. Evidence is presented that the development of the germ cells *in vitro* reflects the presence in the culture medium of the growth and differentiation hormone secreted by the prothoracic glands. The development of the sex cells in the tissue culture appears to be an exceptionally sensitive test for this hormone.

6. By the use of this test the hormone was found to be non-dialyzable and destroyed or precipitated by exposure to temperatures higher than 75° C. Accordingly, it is concluded that the growth and differentiation hormone is either a protein or a smaller molecule conjugated to a protein.

## LITERATURE CITED


BAUMGARTNER, W. J., AND M. A. PAYNE, 1930. Intra vitam technique for the study of the living cells of insects. *Science,* **72**: 199–200.

BÊLÂR, K., 1929. Beiträge zur Kausalanalyse der Mitose. II. Untersuchungen an den Spermatocyten von *Chorthippus* (*Stenobothrus*) *lineatus* Panz. *Arch. f. Entwmech.* **118**: 359–484.

BODENSTEIN, D., 1938. Untersuchungen zum Metamorphoseproblem. II. Entwicklungsrelationen in verschmolzenen Puppenteilen. *Arch. f. Entwmech.,* **137**: 636–660.

CHARIN, N., 1930. Ueber die in vitro Kulturen der Insektengewebe. [In Russian with German summary.] *Trav. Inst. Rech. sci. Univ. Voronèje, No. 4, 103–123.*

DEDERER, P., 1907. Spermatogenesis in *Philosamia cynthia. Biol. Bull.,* **13**: 94–106.

EPHRUSSI, B., AND G. W. BEADLE, 1936. A technique of transplantation for Drosophila. *Amer. Nat.,* **70**: 218–225.

FISCHER, I., AND G. GOTTSCHEWSKI, 1939. Gewebekultur bei *Drosophila. Naturwiss.,* **27**: 391–392.

FRAENKEL, G., 1935. A hormone causing pupation in the blowfly *Calliphora erythrocephala. Proc. Roy. Soc., London, Ser. B,* **118**: 1–12.

FREW, J. C. H., 1928. A technique for the cultivation of insect tissues. *J. Exp. Biol.,* **6**: 1–11.

FUKUDA, S., 1944. The hormonal mechanism of larval molting and metamorphosis in the silkworm. *J. Fac. Sci., Tokyo Imp. Univ., Sec. 4,* **6**: 477–532.

GLASER, R. W., 1917. The growth of insect blood cells *in vitro. Psyche,* **24**: 1–7.

GOLDSCHMIDT, R., 1915. Some experiments on spermatogenesis *in vitro. Proc. Nat. Acad. Sci.,* **2**: 220.

GOLDSCHMIDT, R., 1916. Notiz über einige bemerkenwerte Erscheinungen in Gewebekulturen von Insekten. *Biol. Zentbl.,* **36**: 161–167.

GOLDSCHMIDT, R., 1917. Versuche zur Spermatogenese *in vitro. Arch. f. Zellforsch.,* **14**: 421–450.

GOTTSCHEWSKI, G., AND I. FISCHER, 1939. Ueber das Pigmentausbildungsvermögen von Drosophila-augenanlagen *in vitro. Naturwiss.,* **27**: 584.

LAZARENKO, T., 1925. Amoebocyte syncytia in hanging drops of blood of the beetle, *Oryctes nasicornis. Arch. Mikr. Anat. Forsch.,* **3**: 409–499.

LEWIS, M. R., AND W. R. B. ROBERTSON, 1916. The mitochondria and other structures observed by the tissue culture method in the male germ cells of *Chorthippus curtipennis* Scudd. *Biol. Bull.,* **30**: 99–124.

LÜSCHER, M., 1948. Gewebekultur "in vivo" bei *Rhodnius prolixus* (Hemiptera). *Rev. suisse Zool.,* **55**: 227–232.

MURRAY, M. R., 1926. Secretion in the amitotic cells of the cricket egg-follicle. *Biol. Bull.,* **50**: 210–228.

OMURA, S., 1936. Studies on the reproductive system of the male of *Bombyx mori. J. Fac. Agric. Hokkaido Imp. Univ., Sapporo,* **38**: 151–180.

PLAGGE, E., 1938. Weitere Untersuchungen über das Verpuppungshormon bei Schmetterlingen. *Biol. Zentbl.,* **58**: 1–12.

RIS, H., 1949. The anaphase movement of chromosomes in the spermatocytes of the grasshopper. *Biol. Bull.,* **96**: 90–106.

SCHÜRFELD, W., 1935. Die physiologische Bedeutung der Versondrüsen, untersucht im Zusammenhang mit ihrem feineren Bau. *Arch. f. Entw.,* **133**: 728–759.

STERN, C., 1940. Growth *in vitro* of the testes of *Drosophila. Growth,* **4**: 337–382.

TAYLOR, A., 1935. Experimentally induced changes in the cell complex of the blood of *Periplaneta americana. Ann. Ent. Soc. Amer.,* **28**: 135–145.

THIMANN, K. V., 1952. Plant growth hormones. *In:* The action of hormones in plants and invertebrates. K. V. Thimann, Ed., Academic Press, N. Y.: 1–76.

TRAGER, W., 1935. Cultivation of the virus *grasserie* in silkworm tissue cultures. *J. Exp. Med.*, **61**: 501–513.

WILLIAMS, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm, *Platysamia cecropia*. *Biol. Bull.*, **90**: 234–243.

WILLIAMS, C. M., 1947. Physiology of insect diapause. II. Interaction between the pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, *Platysamia cecropia*. *Biol. Bull.*, **93**: 89–98.

WILLIAMS, C. M., 1952a. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull.*, **103**: 120–138.

WILLIAMS, C. M., 1952*b*. Morphogenesis and the metamorphosis of insects. *Harvey Lectures*, **47**: 126–155.

# THE PHYSIOLOGY OF INSECT DIAPAUSE. VI. EFFECTS OF TEMPERATURE, OXYGEN TENSION, AND METABOLIC INHIBITORS ON *IN VITRO* SPERMATOGENESIS IN THE CECROPIA SILKWORM [1]

HOWARD A. SCHNEIDERMAN,[2] MELVIN KETCHEL AND CARROLL M. WILLIAMS

*The Biological Laboratories, Harvard University, Cambridge 38, Massachusetts*

Throughout the previous papers of this series of studies of the Cecropia silkworm it has been necessary to infer the metabolic reactions of the insect's tissues from the effects of various agents and experimental conditions on the whole or subdivided organism. Manifestly, it would be highly desirable to test these findings on an isolated tissue. Fortunately, this objective is now accessible by virtue of the successful *in vitro* culture of the male sex cells, as demonstrated by Schmidt and Williams (1953). As these investigators have shown, the pupal spermatocytes of the insect, when cultured in simple hanging-drop preparations, undergo meiosis followed by a remarkable differentiation into spermatids and spermatozoa.

In the present investigation we have attempted to define the effects of temperature, oxygen tension, and certain metabolic inhibitors on *in vitro* spermatogenesis. Agents were selected for study whose actions on intermediary metabolism had previously been defined.

## Materials and Methods

### *1. Culture methods*

The methods utilized in culturing the spermatocytes were modifications of those described by Schmidt and Williams (1953). In order to block the tyrosine-tyrosinase reaction, several crystals of phenylthiourea (twice recrystallized from the Eastman product) were added to each sample of blood. Pairs of testes were removed from either diapausing or brainless diapausing male pupae and placed in a depression slide containing several drops of hormone-containing blood from a Cecropia pupa that had just initiated adult development. Each testis was then torn open, releasing thousands of spermatocytal cysts into the blood. The resulting suspension was transferred to a sterile test tube and additional active blood added to a volume of two ml. Each such suspension contained about 100 to 300 cysts per drop. The tubes were then plugged with cotton and stored at 2° C. until used. While failing to develop at this low temperature, the cysts remained viable and, at the convenience of the investigator, could be used in experiments during the following week. Individual hanging-drop cultures were prepared by pipetting a

[1] This study was aided by a grant from the U. S. Public Health Service and an Institutional Grant to Harvard University from the American Cancer Society, Inc.

[2] Former Predoctoral Fellow of the Atomic Energy Commission. Present address: Department of Zoology, Cornell University, Ithaca, New York.

drop of cyst suspension, about 20 mm.$^3$ in volume, onto the underside of a coverslip; the latter was then sealed to a depression slide with melted paraffin.

In experiments where numerous cultures were required, the procedure was altered, as follows. Glass plates, 5″ × 5″ × 0.06″, were covered with thin films of petrolatum and sterilized. "Sitting-drops" of about 20 mm.$^3$ volume were pipetted in rows on each plate. A brass ring, 4″ in diameter × 0.125″ wide × 0.08″ thick, was placed on the glass plate surrounding the drops, and another 5″ × 5″ × 0.06″ glass plate set on top of the ring. The chamber, thus formed, was sealed with

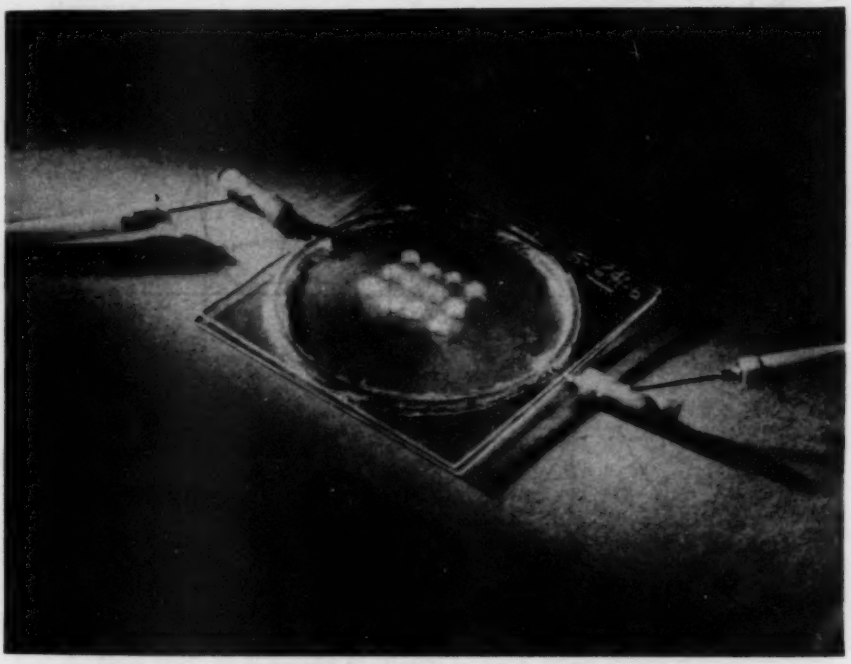


FIGURE 1. Culture chamber for studying the effects of various gases on sitting-drop preparations.

melted paraffin. In experiments in which cysts were exposed to specific gaseous environments, the culture chambers were modified by the addition of inlet and outlet tubes to permit the circulation of suitable gas mixtures (Fig. 1).

*2. Quantitative determination of the rate of spermatogenesis in a population of cysts* [3]

When suspended in blood containing the growth and differentiation hormone ("active blood"), the spermatocytes undergo meiosis and spermiogenesis during

[3] We gratefully acknowledge the assistance of Mr. Ned Feder in the development of this quantitative method.

the course of about two weeks. Most of the cysts contain fully differentiated spermatozoa by the end of this period. In order to quantitate the rate of spermatogenesis, the stage of development of each cyst in the hanging- or sitting-drops was recorded at frequent intervals during the period of culture.

Four successive stages in the development are easily recognized (see Fig. 1, Schmidt and Williams, 1953). Stage I includes primary and secondary *spermatocytal* cysts. Stage II includes spherical *spermatidal* cysts that have not yet begun to elongate. Stage III includes partially elongated *spermatidal* cysts less than twice as long as wide. Stage IV includes elongated *spermatidal* cysts more than twice as long as wide.

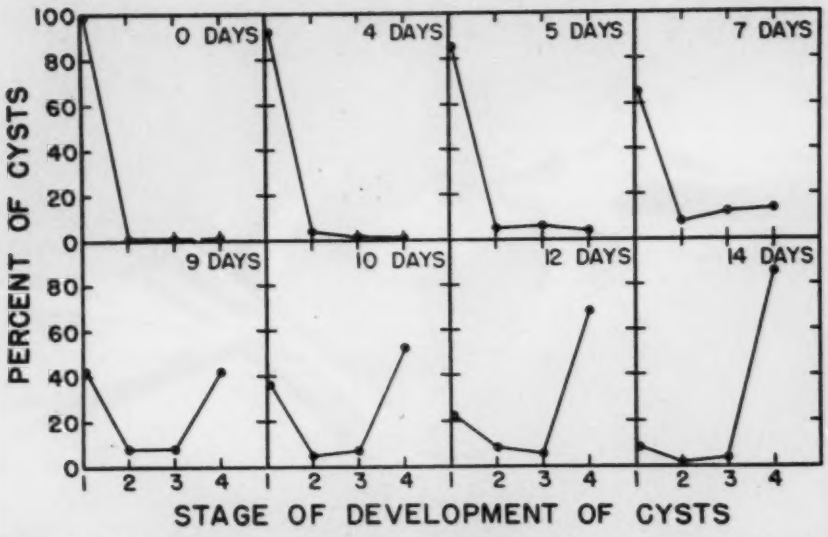



FIGURE 2. Day-to-day profiles during the normal development of a population of 600 cysts. For explanation see text.

As soon as a culture was prepared, the cysts were counted and identified under the low power objective of the compound microscope. This procedure was repeated at one- to three-day intervals. The results, when plotted, produced a series of "profiles" which described the composition of a particular population of cysts as a function of time.

The procedure is illustrated in Figure 2, which records the day-to-day profiles during the normal development of a population of approximately 600 cysts distributed in three hanging-drops. At zero days there was no development. But after five days, 19 per cent of the cysts had undergone meiosis and, of these, about 5 per cent had progressed to stage IV. Thereafter, the percentage of cysts in stage I continued to drop rapidly while that in stage IV increased. After nine days, the two stages were present in nearly equal numbers; after fourteen days, 86 per cent of the cysts had progressed to stage IV.

The determination of the relative rates of spermatogenesis (including both meiosis and spermiogenesis) under various experimental conditions was ordinarily carried out when 30 to 40 per cent of the cysts in a parallel control population had reached stages III or IV. The per cent of cysts in stages III or IV in the experimental population was compared with the corresponding determination on the control population. The relative rate of spermatogenesis was calculated as per cent of that observed in the control.

*3. Reagents*

Stock solutions were prepared in distilled water and the pH adjusted to 6.8–7.2 by the addition of NaOH or HCl. All heat-stable reagents were autoclaved before use. Heat-labile reagents were prepared in sterile distilled water and neutralized with sterile solutions of acid or base.

*4. Gases*

Compressed gases were obtained in commercial cylinders and assayed as follows:

Nitrogen (Airco), 99.5% nitrogen plus less than 0.5% oxygen. Nitrogen (Linde), 99.99% nitrogen plus less than 0.01% oxygen. Oxygen (Airco), 99.5% oxygen plus less than 0.5% nitrogen. Carbon monoxide (Matheson Co.), 96.8% carbon monoxide, 0.36% carbon dioxide, 0.97% hydrogen, 1% nitrogen, 0.8% saturated hydrocarbons, 1.19 mg. iron/liter, 0.32 mg. sulfur/liter. Prior to its use, the carbon monoxide was filtered through 10% sodium hydroxide solution to remove the carbon dioxide and the iron carbonyl compounds.

Mixtures of oxygen, nitrogen, and carbon monoxide were prepared by compression in steel cylinders.

*5. Use of inhibitors*

A fresh suspension of cysts was prepared in active blood and divided into 0.09-ml. aliquots in small test tubes. To each tube was then added 0.01 ml. of an appropriate concentration of an inhibitor. The resulting 0.1-ml. mixture was drawn into a pipette and distributed in five drops of about 20 $mm.^3$ each on a sterile $5'' \times 5''$ glass plate, as described in section 1. The preparations were then maintained at 25° C. in an incubator, and profile counts performed at intervals of one to three days. Three drops were selected containing a total of 300 to 600 cysts. The stage of development of each cyst was scored. Cysts and blood from the same series of donors were used in the preparation of parallel control cultures to which nothing save phenylthiourea was added. In the experimental results, the concentrations of the various inhibitors are recorded as the initial concentrations in the cultures. No attempt was made to define their distribution; *i.e.*, binding to cells, volatilization, or possible loss through degradation.

*6. Addition of gases*

About ten drops of a fresh cyst suspension were enclosed in the chamber illustrated in Figure 1. The glass plates were sealed with petrolatum and held together with clamps (not shown in the figure). Fifty to seventy-five volumes of

an appropriate gas mixture saturated with water vapor were flushed through the chamber; the latter was then submerged in a water bath at 25° C. In experiments utilizing extremely low oxygen tensions, the chambers were flushed out daily to prevent the respiration of the cells from diminishing the oxygen tension significantly.

### *7. Photo-reversibility of carbon monoxide inhibition*

In studies of the photo-reversibility of the carbon monoxide inhibition of spermatogenesis, a 150-watt incandescent lamp was utilized. The light was gathered by a reflector and focussed by means of a biconvex lens; it was then passed through a No. 3962 Corning filter and several centimeters of water to cut off the infrared. Each chamber was submerged in a 25° C. water bath and illuminated from above by a separate lamp. Control chambers were maintained in darkness by an envelope of aluminum foil.

## Results

Preliminary experiments were performed with each agent to define the range of concentration which inhibited spermatogenesis. Concentrations greater than $10^{-2}$ *M* were not investigated unless a specific activity at higher concentrations had previously been reported. A total of approximately 200 experiments was performed; each concentration of each agent was tested in duplicate or triplicate.

Table I

*The effects of temperature on in vitro spermatogenesis*

| Temperature | Time required for initiation of development |
|---|---|
| 10° C. | No development after 31 days |
| 15° C. | 23 days |
| 20° C. | 7 days |
| 25° C. | 3½ days |
| 30° C. | 3½ days |

### *1. Effects of temperature*

The results of the temperature studies are presented in Table I. When cysts were cultured at 10° C., no development was evident after 31 days. At 20° C. the first signs of development were detectable after 7 days, while at 25° C. and 30° C., development began in 3½ days. It is also of interest that after culture at 10° C. for as long as 15 days, the cysts resumed the normal tempo of development when returned to 25° C.

### *2. Effects of metabolic inhibitors*

Figure 3 illustrates the results of a typical experiment in which suspensions of cysts were exposed for seven days to graded concentrations of an inhibitor—in this particular case, 2, 4-dinitrophenol. Development in the presence of $10^{-7}$ *M* proceeded at the same rate as that of controls; in $10^{-6}$ *M* there was definite inhibition; in $10^{-5}$ *M* only a few cysts underwent differentiation; in $10^{-4}$ *M* the cells were killed.

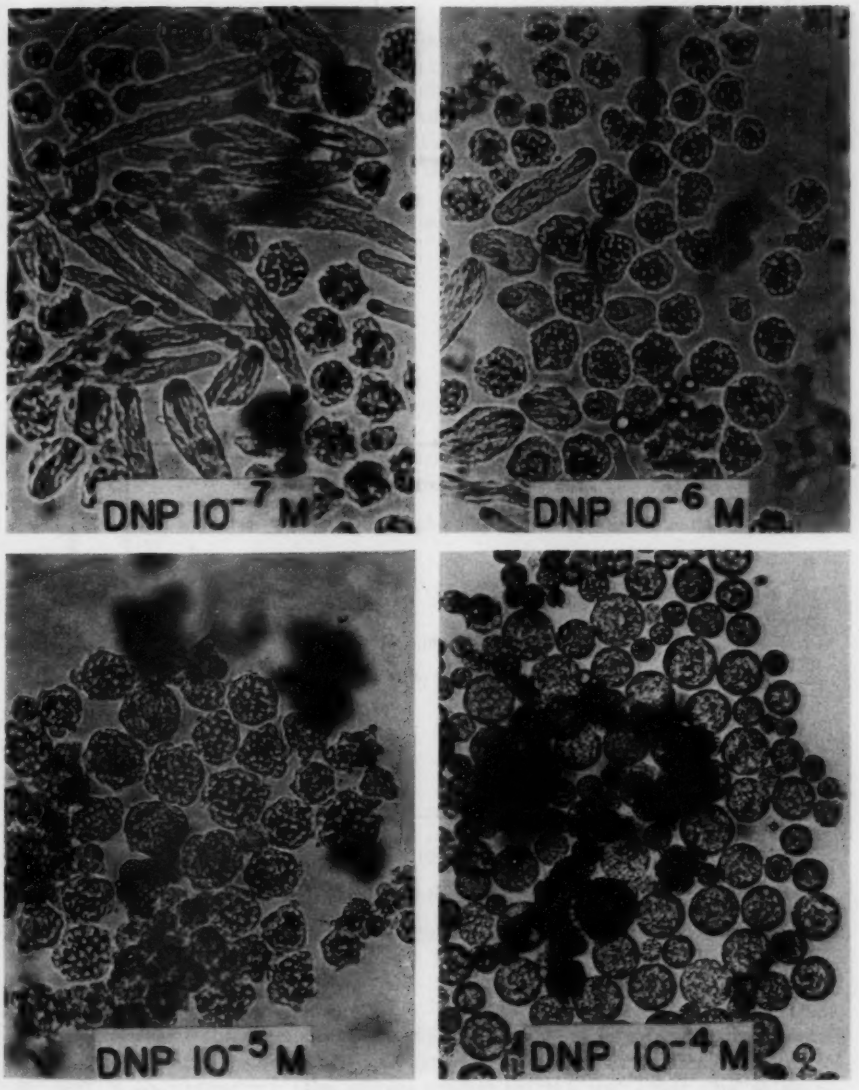



FIGURE 3. Fresh unstained cysts after seven days of culture in graded concentrations of 2, 4-dinitrophenol. × 65.

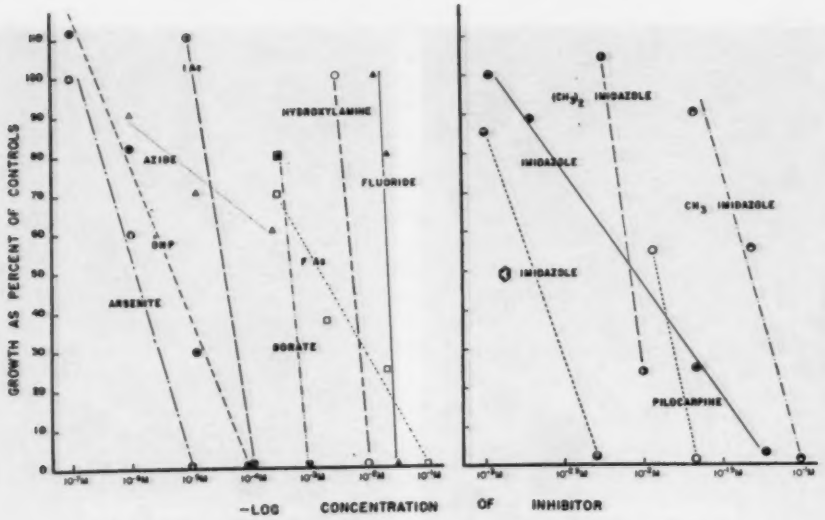



FIGURE 4. Effects of metabolic inhibitors on *in vitro* spermatogenesis.

TABLE II

*The effects of twenty metabolic inhibitors on in vitro spermatogenesis (pH 6.8 to 7.2)*

| Inhibitor | | Approximate concentration required for 50% inhibition of spermatogenesis ($\times 10^{-5}$ *M*) |
|---|---|---|
| 1. Sodium arsenite | | 0.2 |
| 2. 2,4-dinitrophenol | | 0.7 |
| 3. Sodium iodoacetate | | 4 |
| 4. Sodium tetraborate | | 30 |
| 5. Sodium azide | | 80 |
| 6. Sodium fluoroacetate | | 80 |
| 7. Benzimidazole | | 200 |
| 8. Hydroxylamine hydrochloride | | 300 |
| 9. Semicarbazide hydrochloride | | 500 |
| 10. Imidazole | | 700 |
| 11. Dimethyl imidazole | | 700 |
| 12. Pilocarpine | | 1000 |
| 13. Sodium fluoride | | 3000 |
| 14. Methyl imidazole | | 5000 |
| 15. Ferrideuteroporphyrin | | 0.02 mg./ml. |
| 16. Sodium pyrophosphate | No inhibition at | 100 |
| 17. Coumarin | Slight inhibition at | 500 |
| 18. Phenylthiourea* | No inhibition at | 1000 |
| 19. Sodium malonate and malonamide | No inhibition at | 5000 |

* All cultures were saturated with this drug for the purpose of blocking tyrosinase.

The quantitative results of a large number of similar experiments with different inhibitors are summarized in Figure 4. Development is recorded as per cent of that observed in control cultures as a function of the logarithm of the concentration of inhibitor. Although the range of effective concentrations differed considerably in the case of the several inhibitors, for each agent there appeared to be a roughly linear decrease in the rate of spermatogenesis as a function of the logarithm of inhibitor concentration. Table II tabulates the approximate concentrations of 20 chemicals that inhibited the rate of spermatogenesis by 50 per cent.

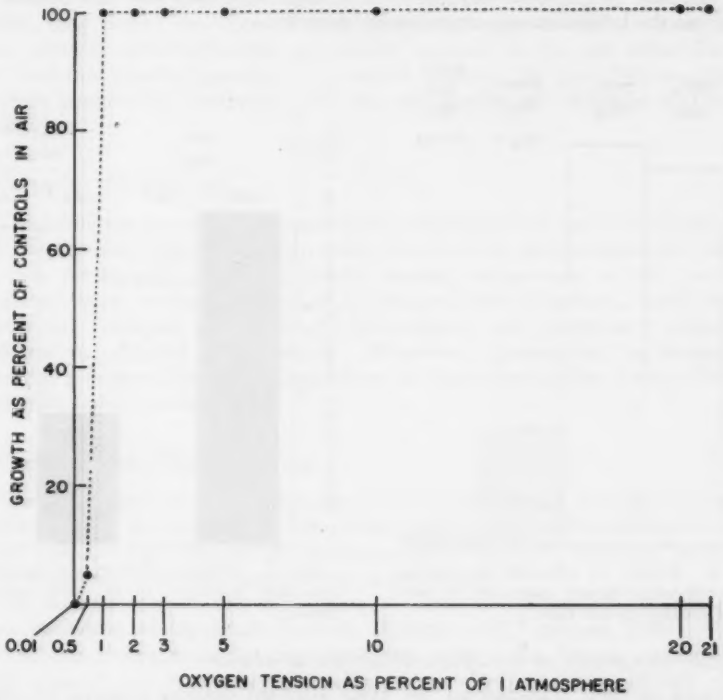



FIGURE 5. Diagrammatic summary of the effects of oxygen tension on *in vitro* spermatogenesis.

### *3. Effects of oxygen tension*

Experiments testing the effects of oxygen tension on *in vitro* spermatogenesis are diagrammatically summarized in Figure 5. Oxygen at tensions between 21 per cent of an atmosphere (the normal tension in air) and 1 per cent of an atmosphere permitted spermatogenesis to proceed at the normal rate. As the oxygen tension was decreased below 1 per cent, considerable inhibition was observed. In 0.5 per cent oxygen the rate of development was only about 5 per cent of normal; in 0.01 per cent oxygen, development was completely inhibited.

*4. Effects of carbon monoxide*

Figure 6 records the effects of carbon monoxide on spermatogenesis. It will be observed that the inhibition depended not only on the pressure of carbon monoxide, but also on the pressure of oxygen that was simultaneously present. Thus, while a mixture of 90 per cent carbon monoxide plus 10 per cent oxygen (9:1) retarded the rate of development by 70 per cent, 90 per cent carbon monoxide plus 5 per cent oxygen (18:1) caused a 98 per cent inhibition.

In another series of experiments, cysts were exposed to 90 per cent carbon monoxide plus 10 per cent oxygen for five days and then returned to air. In many cysts the inhibition was immediately reversed.

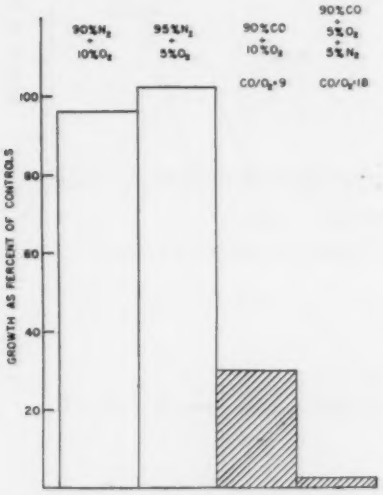



FIG. 6. Effects of exposure to various carbon monoxide/oxygen ratios on *in vitro* spermatogenesis in the dark.

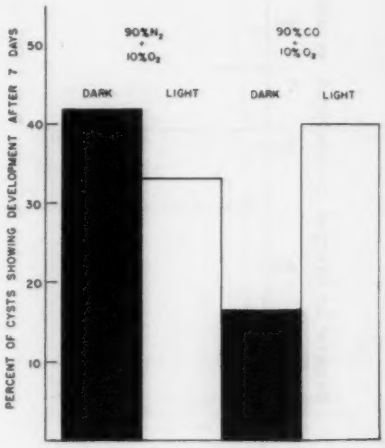



FIG. 7. Effects of light on the carbon monoxide inhibition of *in vitro* spermatogenesis.

*5. Photo-reversibility of the effects of carbon monoxide*

Because of their transparency the cysts afforded optimal material for testing the light reversibility of the carbon monoxide inhibition. Four chambers were used containing, respectively, 90 per cent nitrogen in the light, 90 per cent nitrogen in the dark, 90 per cent carbon monoxide in the light, and 90 per cent carbon monoxide in the dark. Ten per cent oxygen was present in all chambers. The results, recorded in Figure 7, show that light completely reversed the inhibitory effects of carbon monoxide.

## DISCUSSION

The experimental results are relevant to considerations of the intermediary metabolism of both meiosis and spermiogenesis and to previous findings on intact insects.

*1. Temperature*

*In vitro* spermatogenesis showed substantially the same response to temperature as was encountered in the adult development of the animal as a whole. Both proceed most rapidly at environmental temperatures of 25° to 30° C. (Williams, unpublished observations) and are reversibly inhibited by low temperatures.

*2. Glycolytic system*

Though the anti-glycolytic agents, fluoride and iodoacetate, are not wholly specific, their potent inhibition of spermatogenesis suggests that the usual hexose-triose pathway of carbohydrate metabolism operates in the sex cells. However, it is clear that spermatogenesis is an aerobic process; the glycolytic reactions are therefore presumably concerned with the mobilization of substrates for oxidative breakdown.

*3. Tricarboxylic acid cycle*

The inhibition by arsenite, fluoroacetate, hydroxylamine, and iodoacetate is good evidence that the tricarboxylic acid cycle is utilized in spermatogenesis. In intact Cecropia pupae and developing adults, several components in this cycle have previously been demonstrated; namely, succinic dehydrogenase, malic dehydrogenase, and fumarase (unpublished observations) and coenzyme I (Jencks and Williams, unpublished observations). Moreover, fluoroacetate is known to be extremely poisonous for intact Cecropia at all stages in the life history (Williams, unpublished observations).

*4. Phosphate bond energy*

The inhibition by low concentrations of 2,4-dinitrophenol strongly suggests that spermatogenesis is energized by phosphate bond energy. This conclusion is consistent with the high toxicity of 2,4-dinitrophenol for intact Cecropia at all stages of development (Williams, unpublished observations).

We likewise suspect that the toxicity of azide is attributable, at least in part, to its inhibition of phosphate fixation (Loomis and Lipmann, 1949). Though azide is also known to inhibit the cytochrome system, the drug is extremely toxic when injected into diapausing Cecropia pupae (Williams, unpublished observations), where the function of the cytochrome system is not prerequisite to survival (Schneiderman and Williams, 1952).

*5. Terminal oxidases*

a. The experiments testing the effects of oxygen tension demonstrate that spermatogenesis is an aerobic process which apparently cannot be energized by glycolytic reactions. The short distances for the diffusion of oxygen in the *in vitro* preparations enabled the gas to saturate the terminal oxidase when the ambient gas phase contained oxygen at a tension of only 8 mm. of Hg. This finding contrasts sharply with that observed on the intact insect where oxygen is required to diffuse considerable distances through the tracheal system. Consequently, the

adult development of Cecropia is inhibited when the ambient oxygen tension falls below 38 mm. Hg (5 per cent of an atmosphere) (unpublished observations). The fact that spermatogenesis proceeds at finite, though extremely low, tensions of oxygen is presumptive evidence that the terminal oxidase is cytochrome oxidase—an enzyme which, unlike other oxidases, is saturated at oxygen tensions between 0.25 and 2.5 mm. Hg (Winzler, 1941).

b. The failure of phenylthiourea, a powerful inhibitor of tyrosinase, to block spermatogenesis is in agreement with observations on intact Cecropia. Here phenylthiourea inhibits neither adult development (Williams, unpublished observations) nor respiration (Sussman, 1952). It seems safe to conclude that tyrosinase is not a terminal oxidase in processes responsible for the development of Cecropia *in vitro* or *in vivo*.

c. According to Pappenheimer and Williams (1952), certain imidazoles such as pilocarpine exert their effects by complexing ferriprotophyrin, thereby making this prosthetic group unavailable for the synthesis of heme-containing enzymes such as the cytochromes. The inhibitory action of these imidazoles on spermatogenesis (Fig. 4) roughly parallels their effects in blocking the development of the intact insect.

d. The inhibition of spermatogenesis by ferrideuteroporphyrin is probably attributable to its competitive inhibition of ferriprotoporphyrin (Pappenheimer and Williams, 1952). This inference once again points to the role of enzymes containing ferriprotoporphyrin in the metabolism of spermatogenesis; *e.g.*, certain components of the cytochrome system such as cytochrome *b*. The effects observed *in vitro* are analogous to those observed in intact Cecropia where ferrideuteroporphyrin inhibits adult development and other processes requiring cytochrome-mediated respiration.

e. Carbon monoxide's inhibition of spermatogenesis directly parallels its action on intact Cecropia during the period of adult development (Schneiderman and Williams, 1952). Since *in vitro* spermatogenesis was resumed upon return to air, it is clear that carbon monoxide blocked or retarded development without killing. The carbon monoxide inhibition of spermatogenesis was dependent on the carbon monoxide/oxygen ratio. The same finding has been demonstrated for the inhibition of adult development in the intact insect. Finally, the carbon monoxide inhibition of *in vitro* spermatogenesis was reversed by light—an effect which we have duplicated in the intact insect and shall describe in a subsequent communication.

Only one substance is affected by carbon monoxide in this manner; namely, cytochrome oxidase. We therefore conclude that cytochrome oxidase is the terminal oxidase in the morphogenesis of the testicular tissue, as in the insect as a whole.

The photograph in Figure 1 was made in collaboration with Dr. Roman Vishniac and is used with the permission of *Time*, Inc.

## Summary

1. The effects of temperature, metabolic inhibitors, oxygen tension, and carbon monoxide were studied in relation to the *in vitro* spermatogenesis (meiosis and spermiogenesis) of the male sex cells of the Cecropia silkworm.

2. A method is described for the quantitative study of the rate of spermatogenesis.

3. The optimum temperature for spermatogenesis is 25° to 30° C.

4. An appraisal of the effects of twenty diverse metabolic inhibitors revealed that the glycolytic system mobilizes substrates for spermatogenesis, that the tricarboxylic acid cycle is apparently the main pathway of acetate utilization, and that oxidative phosphorylation energizes spermatogenesis.

5. Spermatogenesis is shown to be an aerobic process, the terminal oxidase mediating the respiration being saturated by oxygen at tensions lower than 8 mm. of Hg.

6. Spermatogenesis is blocked or retarded by several inhibitors of the cytochrome system; the inhibition by carbon monoxide is light-reversible.

7. The findings on the isolated sex cells are compared to the effects of the same series of agents on the intact insect. The biochemical systems responsible for the meiosis and differentiation of spermatocytes *in vitro* are apparently the same as those which serve the development of the insect as a whole.

## LITERATURE CITED

LOOMIS, W. F., AND F. LIPMANN, 1949. Inhibition of phosphorylation by azide in kidney homogenates. *J. Biol. Chem.*, **179**: 503–504.

PAPPENHEIMER, A. M., JR., AND C. M. WILLIAMS, 1952. The effects of diphtheria toxin on the *Cecropia* silkworm. *J. Gen. Physiol.*, **35**: 727–740.

SCHMIDT, E., AND C. M. WILLIAMS, 1953. Physiology of insect diapause. V. Assay of the growth and differentiation hormone of Lepidoptera by the method of tissue culture. *Biol. Bull.*, **105**: 174–187.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1952. The terminal oxidases in diapausing and non-diapausing insects. *Anat. Rec.*, **113**: 55–56.

SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1953. Physiology of insect diapause. VII. The respiratory metabolism of the *Cecropia* silkworm during diapause and development. *Biol. Bull.*, in press.

SUSSMAN, A., 1952. Tyrosinase and the respiration of pupae of *Platysamia cecropia* L. *Biol. Bull.*, **102**: 39–47.

WINZLER, R. J., 1941. The respiration of baker's yeast at low oxygen tension. *J. Cell. Comp. Physiol.*, **17**: 263–276.

# CHOLINESTERASE AND LIPASE IN THE AMOEBOCYTES, INTESTINAL EPITHELIUM AND HEART MUSCLE OF THE QUAHOG, VENUS MERCENARIA [1]

SUMNER I. ZACKS [2] AND JOHN H. WELSH

*Department of Anatomy, Harvard Medical School, and Department of Biology, Harvard University, Cambridge 38, Mass.*

The isolated heart of the quahog, *Venus mercenaria,* has been shown by Prosser (1940) to be highly sensitive to acetylcholine and has been used by Welsh (1943) for the biological assay of acetylcholine. This preparation is particularly useful due to its great sensitivity to acetylcholine, resistance to changes in pH and rapid recovery. Thresholds for inhibition of the spontaneous beat of the isolated heart by acetylcholine are of the order of $10^{-10}$ to $10^{-11}$ gm./ml. Treatment of the heart with cholinesterase inhibitors (physostigmine, prostigmine or di-isopropyl fluorophosphate) results in a two to five times increase in inhibition of the strength of the heart beat when acetylcholine is added subsequently (Welsh and Taub, 1948). This slight potentiation is attributed to the low cholinesterase activity reported in the *Venus* heart by Jullien *et al.* (1938) and Smith and Glick (1939).

Augustinsson (1946a) showed that the tissues of the mollusc *Mya arenaria* hydrolyzed acetylcholine, benzoylcholine and acetyl $\beta$ methyl choline and that the blood of the snail *Helix pomatia* hydrolyzes these substrates (1946b). Mendel, Mundell and Rudney (1943) reported that benzoylcholine is hydrolyzed by serum cholinesterase [3] but not by acetylcholinesterase or esterase and that acetyl-$\beta$-methyl choline is hydrolyzed only by acetylcholinesterase.

In a series of unpublished experiments concerned with the staining of the *Venus* heart with supravital dyes, questions arose regarding the localization of esterase activity in the heart, and the association of esterase activity with cell components which stained with Janus green B.

After staining whole *Venus* hearts in Janus green B 1 : 10,000 in sea water for two hours at 19° C., the ventricular and atrial muscle fibers were stained light blue-green. The cut end of the intestine was intensely stained, but little staining was observed in the circular muscle of the intestine. Scattered throughout the heart muscle, numerous amoebocytes were filled with dark blue-green granules. Reduction of the blue-green dye occurred when fragments of atrial muscle were spread under a cover slip on a microscope slide. The blue-green color of di-ethyl safranin dimethyl anilin (Janus green B) changed to the pink-red color of di-ethyl safranin,

[1] This work was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[2] Jeffries Wyman Scholar, Harvard University, 1952–53.

[3] Serum cholinesterase is synonymous with non-specific cholinesterase, pseudo-cholinesterase, type II cholinesterase, and s-type cholinesterase. Human serum, dog muscle, guinea pig liver, rat liver and numerous other tissues are capable of attacking benzoylcholine at various rates. Acetylcholinesterase is synonymous with specific cholinesterase, type I or true cholinesterase. Esterase is synonymous with non-specific esterase or aliesterase.

the reduction product. This indicates that hydrogen donor enzymes are probably present in the amoebocyte granules.

Structures which are known to contain esterase or cholinesterase and which stain with Janus green B occur in motor end-plates (Couteaux, 1947) and mitochondria (Zacks and Welsh, 1951). The appearance of intercostal muscle end-plates stained supravitally by Janus green B and for cholinesterase is similar. In both cases, the edges of the subneural apparatus of the end-plate are reactive. Rat liver mitochondria, which stain selectively with dilute solutions of Janus green B, also contain serum cholinesterase (Zacks and Welsh, 1951). The basis of this parallelism in staining may be due to the quaternary nitrogen groups of the basic dyes which mimic the "cationic head" of acetylcholine. Other evidence for the specific combination of Janus green B with cholinesterases and "receptor" substance of the *Venus* heart and frog end-plates occurs in the strong inhibitory actions of Janus green B on cholinesterase (Massart and Dufait, 1941) and the blocking activity of Janus green B and other basic dyes in the frog rectus abdominis preparation (unpublished observations).

The Janus green B staining granules of amoebocytes may well be regarded as mitochondria since they stain with this dye, contain hydrogen donor enzymes and stain with mitochondrial stains. Further investigations on the histochemistry of *Venus* amoebocytes will appear elsewhere.

The esterase and lipase activity of the amoebocytes and alimentary tract of several bivalve molluscs has been studied. Yonge (1926b) reported that amoebocytes from the oyster *Ostrea* were able to digest olive oil and fish erythrocytes, thus indicating the presence of intracellular lipase and protease. The ingestion and digestion of olive and peanut-oils were confirmed in the oyster, *Crassostrea virginica*, by George (1952). Takatsuki (1934) found difficulty in following the hydrolysis of olive oil in the amoebocytes of *Ostrea* but observed that methyl acetate was more readily hydrolysed. This finding probably indicates the presence of esterase. Yonge (1923) reported that lipolytic enzymes are absent in the midgut of the clam *Mya*. Details of the physiology and biochemistry of amoebocytes may be found in the work of Yonge (1923, 1926a, 1926b, 1929, 1946), Ohuye (1938), and the review of Haughton (1934).

In view of the early reports of lipase and esterase in amoebocytes and intestine of various molluscs and the selective staining of amoebocyte mitochondria by Janus green B, an investigation of the enzymes present in the isolated *Venus* heart, amoebocytes and intestine was of interest.

This investigation is divided into two parts. The first is concerned with evidence of enzymatic activity in the *Venus* heart and isolated amoebocytes obtained by manometric and biological methods. The second part presents evidence of enzymatic activity obtained through the use of recently developed histochemical methods.

## Part I

### Methods

Five *Venus* hearts were excised with an enclosed length of intestine and blotted to remove surface moisture. The combined net weight was 728 mg. The hearts were then homogenized for 25 seconds in 30 ml. of sea water in a Waring Blendor.

A modified version of the standard (Ammon, 1934) manometric procedure for the determination of cholinesterase activity was used. Warburg vessels with side arms were filled with 3 ml. of a solution composed of 25 ml. sea water, 5 ml. 1.26% $NaHCO_3$ and 0.5 ml. of a solution of acetylcholine bromide 8 mg./ml. or benzoylcholine 8 mg./ml. The final acetylcholine concentration in each vessel was 1 mg./ml. Aliquots (0.5 ml.) of the whole heart homogenate were added to the side arm of the flasks. Another 0.5-ml. aliquot of homogenate was dried at 60° C. for 24 hours and the true dry weight was obtained by subtracting the weight of the salts in the aliquot. Four vessels were filled by the method described above and automatic correction of spontaneous hydrolysis was provided by employing a thermobarometer containing substrate but not homogenate. The vessels were equilibrated 10 minutes at 21° C., gassed with a mixture of 95% nitrogen and 5% carbon dioxide for three minutes and re-equilibrated for another 10 minutes. At the end of this period, the homogenate was tipped in and readings taken at 10-minute intervals for 60 minutes. $Q_{Ach}$ values were calculated on the basis: microliters $CO_2$/mg. dry weight × hr.

## Results

Since the cholinesterase activity of the hearts is variable and the relatively low activity is on the threshold of accurate detection by this procedure, a sample experiment will be reported and the average results considered (Table I).

Table I

| | | Acetylcholine bromide 1 mg./ml. | Benzoylcholine 1 mg./ml. |
|---|---|---|---|
| Vessel | I | 2.2 | 4.6 |
| | II | 9.0 | 7.9 |
| | III | 3.2 | 4.0 |
| | IV | 5.1 | — |
| | | Average: 4.8 ($Q_{Ach}$ 0.61) | 5.5 ($Q_{Bach}$ 0.78) |

As can be seen from Table I, these average values ($Q_{Ach} = 0.61$; $Q_{Bach} = 0.78$) are quite low compared with a $Q_{Ach}$ of 34 for horse serum (Mendel and Rudney, 1943) or the average $Q_{Ach}$ of 1.42 for isolated rat liver mitochondria (Zacks and Welsh, 1951). Also, the average values found in this study are lower than the $Q_{Ach}$ of 5.5 reported by Smith and Glick (1939) calculated on the basis of microliters of $CO_2$/30 minute × mg. weight. According to the criteria of Mendel, Mundell and Rudney (1943), the splitting of benzoylcholine by *Venus* heart homogenates indicates the presence of serum cholinesterase. Acetylcholinesterase and esterase do not attack benzoylcholine according to these authors.

The confirmation of low serum cholinesterase activity in *Venus* heart homogenates was achieved, but the localization of the enzyme in the several tisuses included in the homogenate required further investigation. In practice, a length of intestine as well as numerous amoebocytes is included in the heart preparation used for the assay of acetylcholine and in the whole heart homogenates, so that attention must be directed to these component tissues.

In order to determine if the numerous amoebocytes in the heart contributed to the cholinesterase activity of the homogenates, isolated and washed amoebocytes were prepared and assayed for cholinesterase activity.

Five ml. of *Venus* blood were obtained by ventricular aspiration of several quahogs. The pooled blood was centrifuged at 700 × g. for 10 minutes to sediment the amoebocytes. The supernatant was decanted and stored at 4° C. in the refrigerator until it could be assayed. The sedimented amoebocytes were taken up in sea water and re-sedimented in order to wash away adhering serum. This procedure was repeated and the sediment was diluted with 5 ml. sea water. To 7 ml. of sea water, two ml. amoebocyte concentrate and one ml. $10^{-4}$ *M* acetylcholine bromide were added. This mixture was incubated at 27° C. for two hours and 0.1-ml. aliquots were tested on the *Venus* heart preparation (Welsh and Taub, 1948). Similarly, aliquots of mixtures containing separated serum were also tested. Control incubation mixtures were prepared which contained 6 ml. sea water, two ml. amoebocyte concentrate, one ml. physostigmine salicylate $10^{-3}$ *M* and one ml. acetylcholine bromide $10^{-4}$ *M*. After two hours incubation, mixtures were added to regularly

TABLE II

*Summary of the composition of incubation mixtures used to detect cholinesterase activity*

| Number | (Ach) in mixture | Aliquot ml. | (Ach) in heart bath | Serum in mixture | Amoebocytes in mixture ml. | Physostigmine *M* | % inhibition |
|---|---|---|---|---|---|---|---|
| 1 | — | — | $10^{-8}$ *M* | — | — | — | 100 |
| 2 | $10^{-5}$ *M* | 0.1 | $10^{-7}$* | — | 2 | — | 0 |
| 3 | — | — | $10^{-7}$ | — | — | — | 100 |
| 4 | $10^{-5}$ | 0.1 | $10^{-7}$* | — | 2 | — | 0 |
| 5 | $10^{-5}$ | 0.1 | $10^{-7}$* | — | 2 | $10^{-4}$ | 100 |
| 6 | $10^{-5}$ | 0.1 | $10^{-7}$* | — | 2 | — | 0 |
| 7 | $10^{-5}$ | 0.1 | $10^{-7}$* | — | 2 | — | 0 |
| 8 | $10^{-5}$ | 0.1 | $10^{-7}$* | 2 | — | — | 0 |
| 9 | $10^{-5}$ | 1.0 | $10^{-6}$* | 2 | — | — | 14 |
| 10 | — | — | $10^{-6}$ | — | — | — | 100 |

* If there had been no hydrolysis of acetylcholine.

beating *Venus* hearts in a bath containing 9 ml. sea water. The final concentration of acetylcholine was $10^{-5}$ *M* in the control mixtures. The concentration of acetylcholine added to the incubation mixture was chosen so that the final concentration in the heart bath, $10^{-7}$ *M*, if there had been no hydrolysis of acetylcholine, would produce complete inhibition of the heart beat. In a typical experiment, test concentrations of acetylcholine were added to the bath containing the beating heart to measure the sensitivity of the preparation. After washing and a 5-minute rest period, test aliquots of the incubation mixtures were added to the baths. After washing and rest, control concentrations of acetylcholine were added to the baths to test the recovery of the test hearts.

Table II summarizes the manner in which the various incubation mixtures in a sample experiment were prepared. The effect of the various mixtures on the test hearts can be seen by comparing the number of the mixture in Table II with the corresponding number in Figure 1.

Figure 1 is a typical record of a sample experiment. It can be seen that the amoebocyte concentrate and the supernatant serum produced nearly complete hy-

drolysis of the acetylocholine present in the incubation mixtures in two hours as evidenced by the failure of test aliquots to inhibit the spontaneous beat of the isolated heart. The same final concentration of acetylcholine ($10^{-7}$ *M*) added to the bath and aliquots of incubation mixtures protected with $10^{-4}$ *M* physostigmine salicylate, completely inhibited the beat of the *Venus* hearts tested. Test concentrations of acetylcholine ($10^{-7}$ *M*), added to the test hearts after the experimental aliquots had been tested, showed that the test hearts were still sensitive to acetylcholine.

Thus, isolated and washed *Venus* amoebocytes as well as serum contain enzymes capable of hydrolyzing acetylcholine. The observed complete inhibition of these enzymes by physostigmine salicylate $10^{-4}$ *M* indicates the presence of cholinesterase, since lipase and esterase are slightly inhibited by this concentration of physostigmine (Easson and Stedman, 1937; Richter and Croft, 1942). The presence of lipase or esterase in *Venus* amoebocytes cannot be excluded without additional evi-

FIGURE 1. Sample record of the response of the isolated Venus heart to aliquots of incubation mixtures, to detect the presence of acetylcholine.

dence. The histochemical data, which will be presented below, indicate that a mixture of enzymes exists in the amoebocytes as well as in the intestine and heart muscle.

## PART II

In order to obtain more information concerning the intracellular localization of esterases, recently developed histochemical methods were applied to amoebocytes, intestinal epithelium and heart muscle of *Venus*. These procedures have been used in combination with various enzyme inhibitors in an attempt to characterize the types of enzymes present.

### METHODS

Nachlas and Seligman (1949) developed a procedure for the histochemical localization of esterase and lipase in acetone-fixed tissues. This method is based on the hydrolysis of β-naphthyl acetate by esterase and lipase. When β-naphthyl acetate is hydrolyzed, the resulting product is spontaneously converted to β-naphthol which, in the presence of a stabilized diazotised salt, precipitates as a granular, insoluble pigment at or near the site of enzymatic activity. Later, it was found that β-naphthyl acetate also was attacked by the enzymes of fresh, unfixed nervous tissue and that the failure of nervous tissue to react in the original method was due to the destruction of acetylcholinesterase by acetone. The modified procedure (Ravin, Zacks and

Seligman, 1953) utilizes the 6-brom derivative of $\beta$-naphthyl acetate as the substrate and tetrazotized anisidine (diazo blue B) as the coupling agent in the staining of fresh frozen sections cut by the Linderstrøm-Lang method as modified by Coons *et al.* (1951). Sections cut at 10 micra can be stored at 4° C. for one or two weeks without marked decline in enzymatic activity. When applied to fresh, frozen sections, this method will demonstrate lipase, esterase and cholinesterases. The particular enzyme present is determined by pre-treatment of the sections for one hour in various inhibitors known to differentiate lipase, esterase and cholinesterases.

Sodium taurocholate ($10^{-2}$ *M*, $10^{-3}$ *M*) was used to differentiate lipase from esterase since, according to Willstatter and Memmen (1924) and Nachlas and Seligman (1949), esterase is inhibited, but lipase is activated or unaffected by this reagent. Advantage was taken of the observations that acetylcholinesterase and serum cholinesterase are inhibited by low concentrations of physostigmine ($10^{-5}$ *M*) while esterase is little affected by concentrations as great as $10^{-3}$ *M* (Easson and Stedman, 1937; Richter and Croft, 1942). Also cold acetone (4° C.) applied for 24 hours destroys cholinesterase activity but lipase and esterase activity are relatively unaffected (Nachlas and Seligman, 1949). Other inhibitors which are claimed to differentiate between the various enzymes considered were also used.

Quinine ($5 \times 10^{-2}$ *M*) was used by Nachlas and Seligman (1949) who found that pancreatic hydrolysis of $\beta$-naphthyl acetate was moderately inhibited as was esterase activity in liver and kidney. These authors conclude that quinine, in this concentration, inhibits lipase more than esterase. Also, Augustinsson (1948) reported that quinine hydrochloride ($3.78 \times 10^{-4}$ *M*) strongly inhibited the hydrolysis of choline esters and tributyrin catalyzed by serum and liver preparations. Due to this lack of clearly specific inhibition produced by quinine, this reagent is less valuable for the characterization of the enzymes present in the *Venus* heart. Quinine alkaloid was used in a concentration of $10^{-3}$ *M*.

Arsenilic acid (atoxyl) $10^{-1}$ *M* was used by Nachlas and Seligman (1949) to inhibit esterase in homogenates of rat kidney, liver and pancreas and other tissues. The high concentration required attests to the low specificity of this reagent. Thus, to differentiate the main recognized groups of enzymes (lipase, esterase and cholinesterase), reliance was placed on the use of sodium taurocholate, physostigmine and cold acetone as the most reliable criteria available.

As a check on the accuracy of localization of enzymatic activity demonstrated by the use of 6-brom-2-naphthyl acetate, the method of Barrnett and Seligman (1951) was also used. In this procedure, the substrate employed is indoxyl acetate, which, when hydrolyzed by lipase, esterase or cholinesterase, yields indoxyl which oxidizes in air to form an insoluble precipitate of indigo at or near the site of enzymatic activity. The enzymes which attack this substrate are the same as those which hydrolyze 6-brom-2-naphthyl acetate and therefore must also be controlled by the use of inhibitors.

To test for the presence of serum cholinesterase, the method of Ravin, Tsou and Seligman (1951) was used. This procedure employs carbonaphthoxycholine iodide as the artificial chromogenic substrate. The coupling agent (diazo blue B) is the same as that used with 6-brom-2-naphthyl acetate. Carbonaphthoxycholine is hydrolysed by serum cholinesterase, but not by esterase, lipase or acetylcholinesterase.

The histochemical methods described above were applied to fresh whole hearts from which thin spreads were made of atrial muscle or to frozen sections, cut at 10 micra in the Linderstrøm-Lang cryostat. The sections were incubated in a Michaelis barbital buffer (pH 7.4 for 6-brom-2-naphthyl acetate and carbonaphthoxycholine and pH 8.4 for indoxyl acetate) containing the substrate, diazo blue B, NaCl and $CaCl_2$ as recommended in the respective methods. In inhibition experiments, the inhibitors were made up in the proper buffer mixture and slides were incubated in the solutions for 60 minutes. At the end of this time, the inhibitor solution was poured off into a flask and substrate and coupling agent were added. The mixture was then filtered back into the coplin jar containing the sections. In all cases, the reaction was carried out in the presence of the inhibitor to avoid reversibility of inhibition due to washing out of the inhibitor. The reaction was allowed to proceed for 20 minutes and the slides were washed in tap water and mounted in Kaiser's glycerogel. The slides were examined as soon as possible after staining since deterioration of the preparations occurred.

## Results and Discussion

Table III summarizes the results obtained in the histochemical examination of *Venus* amoebocytes, intestinal epithelium and heart muscle.

Table III

*Hydrolysis of histochemical substrates by Venus amoebocytes, intestinal epithelium and heart muscle*

| Substrate | Amoebocytes | Intestinal epithelium | Heart muscle |
|---|---|---|---|
| 6-brom $\beta$-naphthyl acetate | ++ | ++ | ++ |
| Indoxyl acetate | ++ | ++ | ++ |
| Carbonaphthoxycholine | ++ | + | ++ |

After reaction with 6-brom-2-naphthyl acetate or indoxyl acetate, the amoebocytes were filled with blue or blue-violet punctate granules. Red-violet granules were seen in the amoebocytes after reaction with carbonaphthoxycholine iodide. The intestinal epithelium, especially the middle and basal regions of the tall columnar cells, was strongly reactive in many of the intestines examined as was the ventricular and atrial musculature. In other intestines, relatively little enzymatic activity was observed even though amoebocytes and heart muscle reacted normally. Apparently, these differences of enzyme activity in the intestinal epithelium reflect functional phases probably associated with digestion. Nuclei of amoebocytes, intestinal epithelium cells and heart muscle were never reactive.

Table IV summarizes the results obtained in the inhibition experiments. After pre-treatment with sodium taurocholate ($10^{-3}$ $M$), no inhibition of amoebocytes or intestinal epithelium was observed although enzymatic activity in the ventricular muscle was partially inhibited. When a higher concentration of sodium taurocholate ($10^{-2}$ $M$) was used, the intestinal epithelium and amoebocytes were still reactive, but the ventricular muscle enzymatic activity was completely inhibited. In a few experiments, it appeared that the reaction in the amoebocytes and intestinal epithelium

after sodium taurocholate ($10^{-3}$ *M*) had been increased and that activation of the enzymes present had occurred.

Physostigmine alkaloid ($10^{-3}$ *M*) produced complete inhibition of all reactive tissues, but the same inhibitor tested in lower concentration ($2 \times 10^{-5}$ *M*) failed to inhibit the amoebocytes but did somewhat reduce the intensity of the reaction in the intestinal epithelium and more intensely inhibited enzymatic activity in the ventricular muscle. The results obtained with physostigmine ($10^{-3}$ *M*) should be regarded with some doubt since the addition of diazo blue B to a physostigmine solution of this concentration results in the production of a deep orange solution which suggests that a reaction has taken place which may prevent diazo blue B from subsequently coupling with the substrate.

Acetone (4° C.) applied for 24 hours slightly inhibited the reaction in the intestinal epithelium and amoebocytes but markedly decreased the enzymatic activity of the ventricular muscle.

TABLE IV

*Inhibition of Venus amoebocytes, intestinal epithelium and heart muscle by esterase and lipase and cholinesterase inhibitors*

| Inhibitor<br>Substrate 6-brom β-NA | Amoebocytes | Intestinal epithelium | Heart muscle |
|---|---|---|---|
| Sodium taurocholate $10^{-3}$ *M* | ++ | +++ | + |
| Sodium taurocholate $10^{-2}$ *M* | + | ++ | − |
| Quinine alkaloid $10^{-3}$ *M* | ++ | ++ | + |
| Physostigmine alkaloid $2 \times 10^{-5}$ *M* | ++ | + | ± |
| Physostigmine alkaloid $10^{-3}$ *M* | − | − | − |
| Atoxyl $10^{-1}$ *M* | − | − | − |
| Acetone 24 hr., 4° C. | + | ++ | ± |
| Water 90° C., one min. | − | − | − |

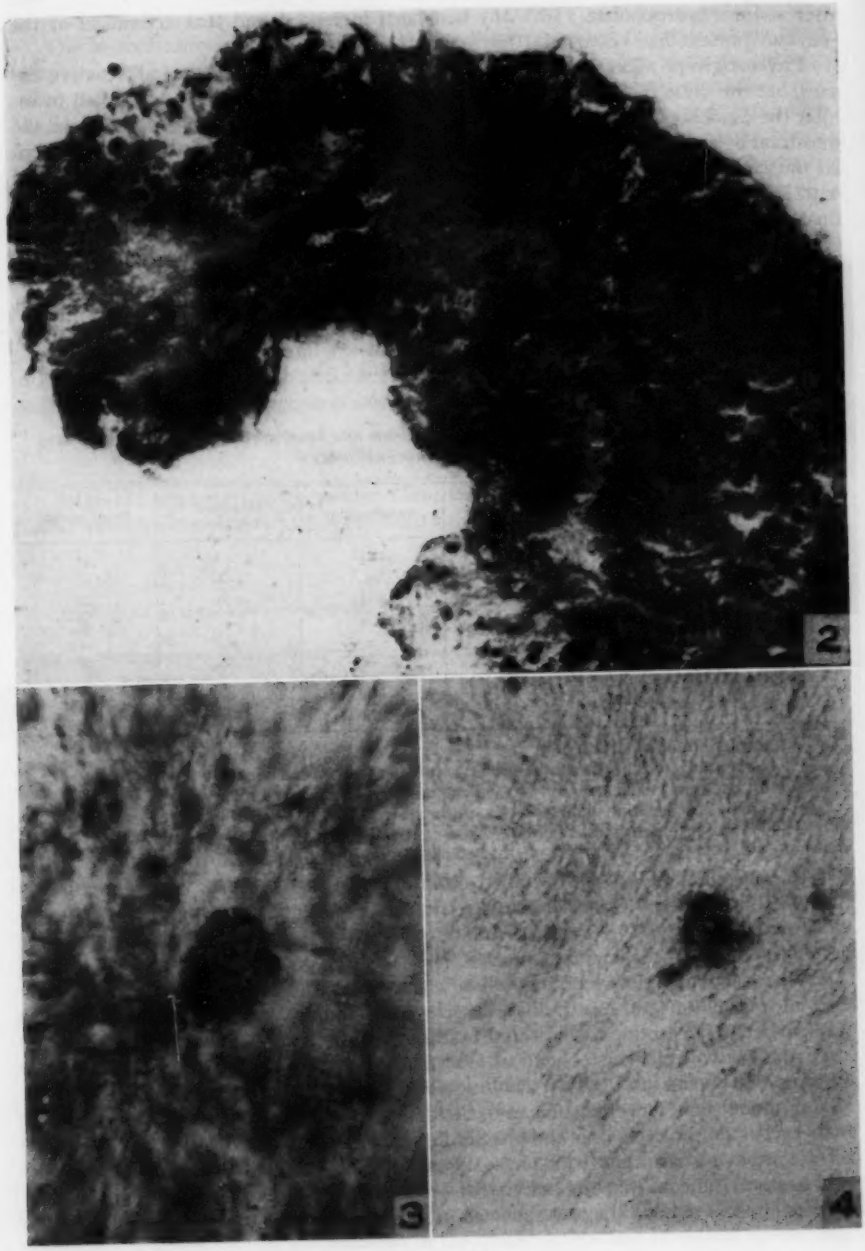
− Complete inhibition.
± Mostly inhibited, but few blue-violet granules present; considerably less than control.
+ Pink background, scattered blue-violet granules; less than control.
++ Fairly intense blue or blue-violet, punctate granulation; control level of staining.
+++ Very intense reaction; greater than control—activation.

Quinine alkaloid ($10^{-3}$ *M*) failed to inhibit the amoebocytes or intestinal epithelium, but slightly decreased the reactivity of the ventricular muscle.

Arsenilate ($10^{-1}$ *M*) completely inhibited all staining in the amoebocytes, intestinal epithelium and ventricular muscle.

When carbonaphthoxycholine iodide was used as the substrate, amoebocytes, intestinal epithelium and ventricular muscle were stained. The ventricular muscle was particularly reactive as indicated by the intense stain.

From the collected histochemical evidence summarized in Tables III and IV, it appears that lipase and serum cholinesterase are present in varying amounts in *Venus* amoebocytes, ventricular muscle and intestinal epithelium. The staining with carbonaphthoxycholine and the inhibition of staining with 6-brom-2-naphthyl acetate after pre-treatment with physostigmine ($2 \times 10^{-5}$ *M*), quinine ($10^{-3}$ *M*) and cold acetone indicate that the activity of serum cholinesterase is greater in the ventricular muscle than in the amoebocytes or intestinal epithelium. Lipase activity is




FIGURES 2–4.

localized in the intestinal epithelium and in the amoebocytes and to a lesser extent in the ventricular muscle. This is shown by the failure of sodium taurocholate to inhibit amoebocyte and intestinal epithelium activity. Ventricular muscle was completely inhibited by $10^{-2}$ *M* sodium taurocholate. Lipase activity in the intestinal epithelium was not destroyed by cold acetone, while the ventricular muscle was completely inhibited.

The histochemical findings summarized above demonstrate the distribution of serum cholinesterase and lipase activity in the *Venus* heart and associated tissues, and account for the results recorded in the literature and in this study. The demonstration of lipase in amoebocytes by histochemical means confirms the observations of Yonge (1926b) who observed the digestion of oil and fish erythrocytes in *Ostrea*. The hydrolysis of acetylcholine and benzoylcholine by whole heart homogenates can be attributed to serum cholinesterase as demonstrated by carbonaphthoxycholine iodide. It has been shown by Ravin *et al.* (1951) that $\beta$ naphthyl acetate is hydrolyzed by homogenates of rat brain, rat liver, washed human erythrocytes and by human serum and purified human serum cholinesterase but that carbonaphthoxycholine iodide is hydrolyzed only by human serum and purified serum cholinesterase. Ravin *et al.* (1951) point out the similarity of carbonaphthoxycholine and benzoylcholine as the basis for the hydrolysis of both substrates by serum cholinesterase. It is interesting that rat liver fails to attack carbonaphthoxycholine yet contains an enzyme capable of hydrolyzing benzoylcholine which is associated with the mitochondria (Zacks and Welsh, 1951). This may indicate that more than one type of serum cholinesterase exists and that the type found in *Venus* is more like the type found in human serum than the enzyme present in rat liver mitochondria. Takatsuki's observation (1934) that amoebocytes of *Ostrea* contain enzymes capable of hydrolysing methyl acetate may be interpreted in terms of serum cholinesterase activity or an esterase. The presence of esterase in the heart and intestinal epithelium of *Venus* can not be completely excluded on the basis of the histochemical experiments. However, the nearly complete protection of acetylcholine by physostigmine ($10^{-4}$ *M*) in incubation mixtures containing isolated amoebocytes suggests that serum cholinesterase is the enzyme in the amoebocytes which attacks acetylcholine most readily.

The role of amoebocytes in intracellular digestion has been studied by Yonge (1923, 1926a, 1926b, 1946) who believes that the digestion of fat and proteins in molluscs is primarily intracellular in the cells of the digestive diverticula and in the

FIGURES 2–4.

FIGURE 2. Fresh frozen transverse section through the intestine of *Venus mercenaria*, showing total enzymatic activity after 20 minutes incubation in a solution containing 10 mg. of 6-brom-2-naphthyl acetate and 40 mg. of diazo blue B in barbital buffer pH 7.4. Note the intense activity in the basal and midportions of intestinal epithelium. The dark globules of "excretory substance" do not contain enzymatic activity but are colored red by dissolved azo dye. Enzymatic activity in the epithelium is indicated by fine blue punctate granules of the precipitated dye. 100 ×.

FIGURE 3. View of a rounded-up amoebocyte, from a fresh spread of atrial muscle, containing granules of precipitated azo dye after 20 minutes staining with a mixture of $\beta$-naphthyl acetate and diazo blue B. 1000 ×.

FIGURE 4. An example of an amoebocyte with a characteristic pseudopod in a fresh spread of atrial muscle stained supravitally by $\beta$-naphthyl acetate and diazo blue B for 20 minutes at room temperature. 1000 ×.

amoebocytes. Thus, the amoebocytes are thought to have a major role in the absorption and digestion of fat and protein. Evidence of extracellular digestion of fats and proteins in the intestinal tract of several molluscs has been obtained by Sawano (1929), Mansour (1946) and Mansour-Bek (1946). George (1952), using Nile blue sulphate as an indicator, observed that oil droplets are hydrolyzed in the stomach of *Crassostrea virginica* and that neutral fat was hydrolyzed by minced crystalline style and digestive gland. This author was also able to confirm the ingestion and subsequent intracellular digestion of oil by the amoebocytes but concluded that the fat ingested was too little to be significant in nutrition.

The demonstration of variable levels of lipase and cholinesterase activity in the intestinal epithelium of *Venus* seems to lend support to the concept of extracellular digestion of fats in the intestinal tract of certain molluscs.

The role of serum cholinesterase, either in sera or tissues, is not known, although a digestive role might be suspected in *Venus* since the enzyme occurs in amoebocytes and intestinal epithelium.

## Summary

1. Whole *Venus* heart homogenates hydrolyze acetylcholine and benzoylcholine at a low rate.

2. The enzymatic activity of such homogenates is the sum of that contributed by amoebocytes, intestinal epithelium and heart muscle.

3. Isolated amoebocytes hydrolyze acetylcholine and the enzyme responsible is inhibited by $10^{-4}$ *M* physostigmine, thus indicating the presence of cholinesterase.

4. Recently developed histochemical procedures demonstrate serum cholinesterase and lipase in amoebocytes, intestinal epithelium and heart muscle.

5. Greatest lipase activity is present in the amoebocytes and intestinal epithelium, while serum cholinesterase activity is greatest in the ventricular muscle.

6. The potentiation of the action of acetylcholine on the isolated *Venus* heart by physostigmine is due to inhibition of serum cholinesterase in the amoebocytes and especially in the heart muscle.

7. The presence of varying levels of lipase in the intestinal epithelium of *Venus* lends support to the suggested presence of extracellular lipolytic enzymes in certain molluscs.

8. In *Venus* amoebocytes, another case is seen where cholinesterase activity is associated with structures which stain supravitally with Janus green B.

## LITERATURE CITED

Ammon, R., 1934. Die fermentative Spaltung des Acetylcholins. *Pflügers Arch. ges. Physiol.*, **233**: 486–491.

Augustinsson, K. B., 1946a. Choline esterases in some marine invertebrates. *Acta Physiol. Scand.*, **11**: 141–149.

Augustinsson, K. B., 1946b. Studies on the specificity of cholinesterase in *Helix pomatia*. *Biochem. J.*, **40**: 343–349.

Augustinsson, K. B., 1948. Cholinesterases, a study in comparative enzymology. *Acta Physiol. Scand.*, **15**: suppl. 52, 1–182.

Barrnett, R. J., and A. M. Seligman, 1951. Histochemical demonstration of esterases by production of indigo. *Science*, **114**: 579–582.

Couteaux, R., 1947. Contribution à l'étude de la synapse myoneurale. *Rev. Canad. de Biol.*, **6**: 565–711.

COONS, A. H., E. H. LEDUC AND M. H. KAPLAN, 1951. Localization of antigen in tissue cells. VI. The fate of injected foreign proteins in the mouse. *J. Exp. Med.*, **93**: 173–188.

EASSON, L. H., AND E. STEDMAN, 1937. The specificity of choline-esterase. *Biochem. J.*, **31**: 1723–1729.

GEORGE, W. C., 1952. The digestion and absorption of fat in lamellibranchs. *Biol. Bull.*, **102**: 118–127.

HAUGHTON, I., 1934. XVII. Amoebocytes and allied cells in invertebrates. *J. Roy. Micr. Soc.*, **54**: 246–262.

JULLIEN, H., D. VINCENT, M. BOUCHET AND M. VIULLET, 1938. Observations sur l'acétylcholine et la choline-estérase du coeur des Mollusques. *Ann. Phys. et Phys. Biol.*, **14**: 567–574.

MANSOUR, K., 1946. Food and digestive organs of lamellibranchs. *Nature*, **158**: 378.

MANSOUR-BEK, J. J., 1946. Extra-cellular proteolytic and lipolytic enzymes of some lamellibranchs. *Nature*, **158**: 378–379.

MASSART, L., AND R. P. DUFAIT, 1941. Hemmung der Acetylcholin-Esterase durch Farbstoffe und durch Eserin. *Enzymologia*, **9**: 364–368.

MENDEL, B., AND H. RUDNEY, 1943. Studies on cholinesterase. 1. Cholinesterase and pseudocholinesterase. *Biochem. J.*, **37**: 59–63.

MENDEL, B., D. B. MUNDELL AND H. RUDNEY, 1943. Studies on cholinesterase. 3. Specific tests for true cholinesterase and pseudocholinesterase. *Biochem. J.*, **37**: 473–476.

NACHLAS, M. M., AND A. M. SELIGMAN, 1949. The histochemical demonstration of esterase. *J. Nat. Cancer Inst.*, **9**: 415–425.

NACHLAS, M. M., AND A. M. SELIGMAN, 1949. Evidence for the specificity of esterase and lipase by the use of three chromogenic substrates. *J. Biol. Chem.*, **181**: 343–355.

OHUYE, T., 1938. On coelomic corpuscles in the body fluid of some invertebrates. X. Some morphological and histological properties of the granulocytes of various invertebrates. *Science Rep. Tohoku Imperial Univ., Series 4 (Biol.)*, **12**: 593–622.

PROSSER, C. L., 1940. Acetylcholine and nervous inhibition in the heart of *Venus mercenaria*. *Biol. Bull.*, **78**: 92–102.

RAVIN, H. A., K. C. TSOU AND A. M. SELIGMAN, 1951. Colorimetric estimation and histochemical demonstration of serum cholinesterase. *J. Biol. Chem.*, **191**: 843–857.

RAVIN, H. A., S. I. ZACKS AND A. M. SELIGMAN, 1953. The histochemical demonstration of acetylcholinesterase in nervous tissue. *J. Pharm. Exper. Therap.*, **107**: 37–53.

RICHTER, D., AND P. G. CROFT, 1942. Blood esterases. *Biochem. J.*, **36**: 746–757.

SAWANO, E., 1929. Studies on the enzymes of *Ostrea circumpicta*. *Science Rep. Tohoku Imperial Univ. Series 4 (Biol.)*, **4**: 327–334.

SMITH, C. C., AND D. GLICK, 1939. Some observations on cholinesterase in invertebrates. *Biol. Bull.*, **77**: 321–322.

TAKATSUKI, S., 1934. On the nature and function of the amoebocytes of *Ostrea edulis*. *Quart. J. Micr. Sci.*, **76**: 379–431.

WELSH, J. H., 1943. Acetylcholine level of rat cerebral cortex under conditions of anoxia and hypoglycemia. *J. Neurophysiol.*, **6**: 329–336.

WELSH, J. H., AND R. TAUB, 1948. The action of choline and related compounds on the heart of *Venus mercenaria*. *Biol. Bull.*, **95**: 346–353.

WILLSTATTER, R., AND F. MEMMEN, 1924. Über die Wirkung der Pankreaslipase auf verschiedene Substrate. *Zeitschr. physiol. Chem.*, **133**: 229–246.

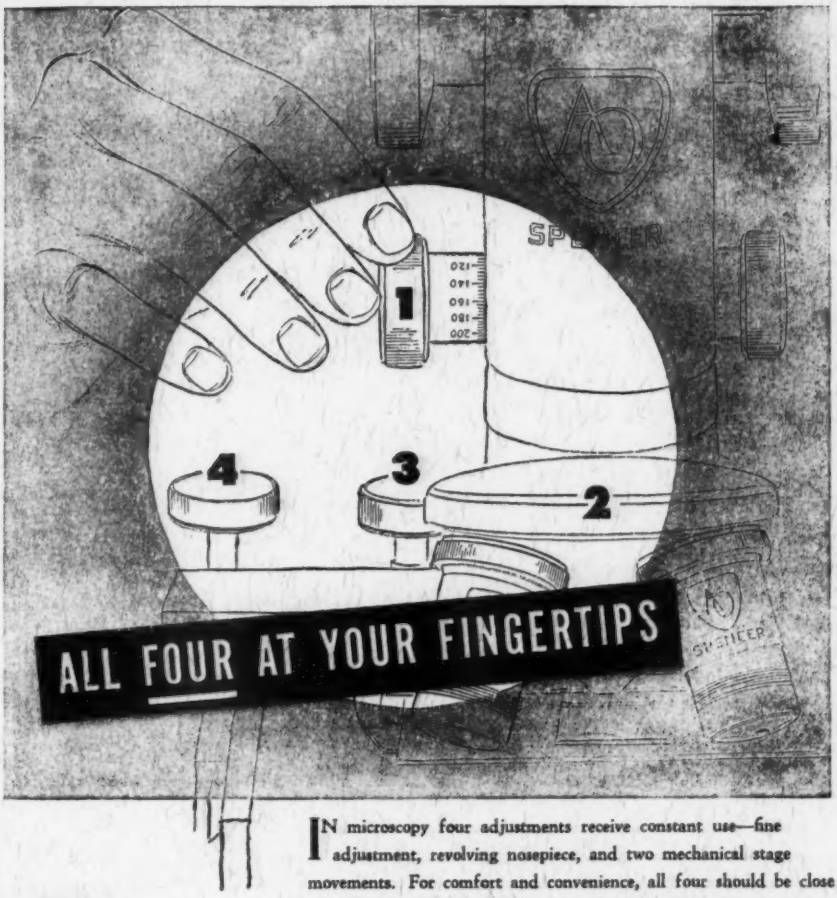
YONGE, C. M., 1923. Studies on the comparative physiology of digestion. Mechanism of feeding, digestion, and assimilation in the lamellibranch, Mya. *Brit. J. Exper. Biol.*, **1**: 15–63.

YONGE, C. M., 1926a. The digestive diverticula in the lamellibranchs. *Trans. Roy. Soc. Edinburgh*, **54**: 703–718.

YONGE, C. M., 1926b. Structure and physiology of the organs of feeding and digestion in Ostrea edulis. *J. Mar. Biol. Assoc.*, **14**: 295–386.

YONGE, C. M., 1946. Digestion of animals by lamellibranchs. *Nature*, **157**: 729.

ZACKS, S. I., AND J. H. WELSH, 1951. Cholinesterases in rat liver mitochondria. *Amer. J. Physiol.*, **165**: 620–623.




IN microscopy four adjustments receive constant use—fine adjustment, revolving nosepiece, and two mechanical stage movements. For comfort and convenience, all four should be close to one another and to the specimen. No matter at what angle you tilt an AO Spencer Laboratory Microscope, these adjustments are adjacent, visible, and so scientifically spaced that fingers glide automatically from one to the other. Even the coarse adjustment is always right at hand.

You'll notice this typical AO *Design Perfection* also in the renowned Spencer Optics, dust-proof, dual-cone nosepiece, "pinch grip" mechanical stage, "autofocus", custom tension adjustment, built-in "full-field" illumination and many other features. But test the many AO Spencer advantages *yourself*. Ask your AO distributor for a demonstration or write Dept. V185.

American Optical

INSTRUMENT DIVISION • BUFFALO 15, NEW YORK

# CONTENTS


Page

ANNUAL REPORT ..... 1

ANDERSON, JOHN MAXWELL
Structure and function in the pyloric caeca of Asterias forbesi ..... 47

CHILD, C. M.
Redox indicator patterns in relation to echinoderm exogastrulation. I. Oxidation patterns ..... 62

FOULKES, ROBERT HUGH
Regeneration of the anterior end of Aulophorus furcatus (Naididae) with special reference to effect of x-rays ..... 80

GOLDSTEIN, LESTER
A study of the mechanism of activation and nuclear breakdown in the Chaetopterus egg ..... 87

HAXO, F. T., AND K. A. CLENDENNING
Photosynthesis and phototaxis in Ulva lactuca gametes ..... 103

HODGSON, EDWARD S.
A study of chemoreception in aqueous and gas phases ..... 115

LOVE, WARNER E.
The permeability of the erythrocyte-like cells of Phascolosoma gouldi .. 128

NANNEY, DAVID L.
Nucleo-cytoplasmic interaction during conjugation in Tetrahymena ... 133

PETTIBONE, MARIAN H.
Fresh-water polychaetous annelid, Manayunkia speciosa Leidy, from Lake Erie ..... 149

ROCKSTEIN, MORRIS
Some aspects of physiological aging in the adult worker honey bee .... 154

ROSENTHAL, HAROLD L.
Embryonic development of the lordotic and normal guppy, Lebistes reticulatus (Peters) ..... 160

SACKTOR, BERTRAM, GERARD M. THOMAS, JOHN C. MOSER AND DONALD I. BLOCH
Dephosphorylation of adenosine triphosphate by tissues of the American cockroach, Periplaneta americana (L.) ..... 166

SCHMIDT, EDMOND L., AND CARROLL M. WILLIAMS
Physiology of insect diapause. V. Assay of the growth and differentiation hormone of Lepidoptera by the method of tissue culture ..... 174

SCHNEIDERMAN, HOWARD A., MELVIN KETCHEL AND CARROLL M. WILLIAMS
The physiology of insect diapause. VI. Effects of temperature, oxygen tension, and metabolic inhibitors on in vitro spermatogenesis in the Cecropia silkworm ..... 188

ZACKS, SUMNER I., AND JOHN H. WELSH
Cholinesterase and lipase in the amoebocytes, intestinal epithelium and heart muscle of the quahog, Venus mercenaria ..... 200